Avian Diseases

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STUDIES ON AVIAN ENCEPHALOMYELITIS. IV EPIZOOTIOLOGY*†

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MOST aspects of the epizootiology of avian encephalomyelitis (AE) have not been clearly defined. The disease has been observed to be extremely wide-spread, being found in all parts of the United States and in many foreign countries. A survey conducted in Connecticut revealed immunity to AE in the vast majority of flocks tested.[8] Taylor and Schelling, in 1960, reported that 95.7 percent of 2,162 flocks tested were immune at 13 to 18 months of age.[9]

Observations of field outbreaks have offered but meager and sometimes confusing information relating to the infectiousness and contagiousness of AE virus. Schaaf and Lamoreux[7] noted from hatchery records that the disease appeared to spread from house to house. Perhaps the chief difficulty in making adequate observations of an outbreak in breeders is the necessity of doing so in retrospect. Since most flocks show no signs of disease other than a possible drop in egg production, an infection generally goes unnoticed until infected chicks are hatched. Natural infections in growing birds are even more difficult to observe and are known to occur chiefly on the basis of serological evidence.

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Conflicting reports exist on the effect of AE on hatchability. Taylor *et al.*, observed both decreased egg production and hatchability coincident with egg transmission of the virus.[10] Jungherr and Minard, on the other hand, reported that hatchability was not affected.[3]

The incidence of clinical signs in chicks from various infected breeder flocks varies considerably.[11] Data is scarce regarding the relationship of incidence in chicks to incidence in their dams, the duration of egg transmission from individual hens, and the occurrence of contact transmission from infected chicks to susceptible contacts. Although Van Roekel has suggested that direct contact is responsible for transmission of the disease,[12] he has further stated that clinical manifestations appear only to a limited degree in non-infected contacts.[11] Schaaf detected some resistance to challenge but no clinical signs in controls held in contact with vaccinated birds.[6]

Neither the source nor the mode of infection has been definitely established Jungherr and Minard[3] were able to demonstrate a virus in tissue and feces from AE-infected adults that produced lesions indistinguishable from those in AE-infected chicks. It has also been established that several routes of inoculation will produce clinical signs, histologic lesions, or immunity to AE[2,5,7], although no one has reported reproducing a syndrome similar to that occuring in field outbreaks in growing or adult birds.

The experiments described herein were done in an attempt to elucidate some aspects of transmission of AE.

MATERIALS AND METHODS

Experimental eggs and birds. All eggs used for virus propagation and serum-neutralization (SN) tests were obtained from two privately-owned flocks of White Leghorns known to be susceptible to AE. Chicks from these flocks were hatched and raised in isolation until used. Adult breeders were obtained from one of the flocks for egg transmission studies. Holding conditions varied among the experiments and included modified Horsfall units, wire batteries, and

pens (floor). Details will be given under the descriptions of individual experiments.

Serology and histopathology. Serum-neutralization tests were conducted and interpreted as previously described.[1] Histopathological examinations were used both to confirm diagnoses in clinically affected birds and to determine presence or absence of infection in clinically normal birds. Tissues examined included brain, gizzard, proventriculus, and pancreas. Infection with AE was considered to have occurred when one or more of the following were observed: clinical signs with positive histopathology, positive histopathology alone, or serological response. In addition to the SN test, embryo susceptibility examinations were used as evidence of immunity status in adult birds. For this test, 6-day-embryos were inoculated via the yolk sac with 100 embryo infective doses—fifty percent (EID_{50}) of egg-adapted AE virus. Previous work (unpublished) indicated that embryos resisting this dosage came from hens with a demonstrable (SN) level of immunity. Eggs from susceptible hens always developed lesions typical of AE as described by Jungherr, *et al.*[4]

Virus strains, titrations, and assay. A total of five AE isolates were used in the work reported here. One of these was the egg-adapted Van Roekel (VR) strain employed for SN tests.[1] Brain suspension from 36th embryo passage was used. Two isolates (28 and 1143) were also embryo-propagated (4th to 8th passage) but produced no lesions in infected embryos. Isolate 774 consisted of brain suspension from clinically affected chicks submitted as a diagnostic case. The fifth isolate, 1288, was used as a pool of brain suspension from AE affected chicks (diagnostic case) and second embryo passage brain. Since only the VR isolate produced observable signs in embryos, assays for virus with the other four isolates necessarily were done in chicks. Brain suspensions of isolates 28 and 1143 were administered in the drinking water to 3 and 4-day-old chicks and were considered to contain viable AE virus upon development of clinical signs, histological lesions, and positive serology. Isolates 774 and 1288 both produced clinical AE in chicks hatched subsequent to yolk-sac inoculation of 6-day-embryos.

Virus assays of feces were conducted in the same manner as with brain suspensions of isolates 28 and 1143. Feces pools were diluted approximately 1:5 in buffered saline and administered in drinking water at a dose level of 1 ml of suspension per chick.

Isolation attempts from affected chicks and embryos pipped, or dead-in-shell (22 days incubation) were done by inoculation of brain suspensions intracerebrally into susceptible day-old chicks. Criteria of infection were as described above.

RESULTS

Experiment 1. In an attempt to produce an active infection of AE in adult birds, three isolates (VR, 28, 1143) were pooled and administered in the drinking water to 34 mated, 6½-month-old White Leghorns. Serum-neutralization tests indicated that 26 of these were susceptible prior to inoculation. The other eight were immune as a result of earlier intramuscular vaccination with VR virus. The birds were held on the floor in a colony house. A virus pool consisting of 5 ml each of 1:5 suspensions of 28 and 1143 and 60 ml of undiluted VR virus were diluted in 12 quarts of tap water and provided as drinking water after 2½ hours water deprivation. Eggs were collected daily for 20 days and set for hatching twice weekly. After hatching, chicks were held in wire batteries in isolation units and observed for 28 days. Data presented in table 1 indicates hatchability and the number of chicks that developed clinical signs of AE.

TABLE I

Egg production, hatchability, and affected chicks following oral administration of AE virus to 34 breeder hens. Experiment 1.

Days Postinoc.	Number Eggs	Embryos Alive 18 da.	% 18 da. emb. hatched	Clinical AE signs
1-2	55	49	94	0/46*
3-6	77	70	97	0/68
7-9	74	68	96	2/65
10-13	89	81	95	0/77
14-16	82	74	95	1/70
17-20	80	72	92	23/66**

* Number affected over number of chicks.
** Thirty-two of forty-three survivors positive histologically at 28 days of age.

The major period of transmission occurred between 17 and 20 days postinoculation, though the first affected chicks were observed in the hatch from eggs laid 7 to 9 days after inoculation. Of 23 clinically affected chicks in the last hatch (eggs laid 17 to 20 days postinoculation), first signs of AE appeared in two chicks at 6 days of age, four at 12 days, two at 14 days, six at 16 days, one at 17 days and three at 20 days. Virus isolation attempts were done on brains of two chicks affected at 14 days of age and one chick affected at 19 days of age. All three yielded AE virus. All chicks affected after the 12th day of age were examined and found to contain lesions of AE in the brain, gizzard, proventriculus and pancreas. Tissues of survivors from the last hatch were examined histologically at 28 days of age. Thirty-two of 43 chicks so examined had lesions.

Experiment 2. This trial was designed to determine the infectivity of the colony house used in experiment 1 for other groups of birds. The pen was not cleaned out and was left vacant for only 48 hours between experiments. A wire partition separated the house lengthwise into halves. All litter was combined and placed on one-half of the floor only. On the other side of the partition, a clean sterilized wire battery was placed. The top deck was approximately 5 feet off the floor and within 2 feet of the wire partition. Attached to the end of the house and directly underneath two large open windows was a wire-floored porch that had been chemically disinfected.

Fully susceptible 12-week-old birds were distributed in the following manner: floor, 20 birds; battery (top deck), 10 birds; and porch, 10 birds. Ten birds were kept in a wire battery in an isolation unit elsewhere to serve as controls. Separate feed cans and feeding and watering implements were used for each group to help avoid mechanical transmission.

Serum-neutralization tests were done on blood samples from 10 birds prior to the start of the experiment. At 3, 5, and 7 weeks postexposure, SN tests were conducted on all groups (excepting isolated controls at 3 weeks). Neutraliza-

tion indices (NI) of 1.1 or greater were considered positive.[2] In the group maintained on the floor, the first positive NI (2.7) was observed at 3 weeks postexposure. By 7 weeks postexposure, 17 of 20 (85 percent) were immune serologically. All birds held in the battery were negative at 5 weeks but four of 10 had become positive by 7 weeks. Birds kept on the porch and in the isolation room remained negative throughout the 7 week period. Data on the serological responses are found in table 2.

Experiment 3. To gain additional information regarding possible modes of infection, a third experiment was conducted using modified Horsfall units. Units 1, 2, and 3 were connected in series with the air inlet attached to unit 1 and the outlet to unit 3. Units 4 and 5 were maintained separately in the usual manner, each having individual inlets and outlets. In order to prevent dust, dropping pans within the units were filled with water and a detergent to a depth of ½ inch.

Chicks for this trial were 19 days of age. Inoculum consisted of the same virus pool used in experiment 1 but diluted in 330 ml of nutrient broth rather than tap water. One ml of virus suspension was pipetted directly into the crop of each of three chicks in unit 1. An additional 3 ml were placed in the drinking water for these birds. Two chicks in each of units 2 and 3 were uninoculated (air contact) controls. Unit 4 held two birds, each given 2 ml of virus suspension in the following manner. Virus was placed within gelatin capsules and was quick frozen. These were then carefully inserted into slightly larger capsules to prevent contamination of the outer surfaces. The double capsules were forced into the crops of the birds which were then restrained in lateral recumbency for a period of 48 hours. The latter was done to avoid oral contamination from feces. The vents of the birds were then cleansed thoroughly with detergent and water and the birds released to a clean sterile unit (unit 4). Unit 5 contained two uninoculated, unexposed controls.

Serum-neutralization tests were conducted on one bird from each unit prior to inoculation and from each bird at 4, 6, and 8 weeks postinoculation. Results are found in table 3.

TABLE II

Serological responses of 12-week-old susceptible birds following various exposures to an AE contaminated colony house. Experiment 2.

	Pre-exposure		Post exposure					
			3 weeks		5 weeks		7 weeks	
Group*	Pos/total	GMT (NI Range)**	Pos/total	GMT (NI Range)	Pos/total	GMT (NI Range)	Pos/total	GMT (NI Range)
Isolated Control	0/10	.3 (–.1 to .8)	not done		0/9	0 (–.2 to .3)	0/4	.1 (–.4 to .5)
Porch			0/5	.4 (.1 to .6)	0/9	–.1 (–.3 to .6)	0/9	–.4 (–.5 to –.2)
Battery			0/5	.3(–.2 to .6)	0/10	.2 (–.3 to .5)	4/10	.9 (–.5 to 2.5)
Floor			1/10	.8 (–.1 to 2.7)	7/20	1.2 (.1 to 2.7)	17/20	1.9 (0 to 2.5)

* Isolated controls = held in isolation unit. Porch = wire floor porch attached to contaminated house. Battery = sterile wire battery within house. Floor = on litter within house. Contamination from AE-infected birds previously held in colony house.

** Positive = Neutralization index (NI) of 1.1 or greater. GMT = geometric mean titer.

TABLE III

Neutralization indices following various exposures of 19-day-old chickens to AE virus. Experiment 3.

Unit*	Exposure per bird	Preinoculation	Postinoculation		
			4 weeks	6 weeks	8 weeks
1	1 ml per crop	–0.1	2.4	2.3	2.0
	1 ml drinking water		2.4	2.3	3.7
			2.5	2.6	4.0
2	air contact**	0.3	0.2	–0.1	–0.1
			0.4	0.7	0.7
3	air contact	–0.1	0.2	–0.4	0.3
			0.2	0.4	0.7
4	2 ml (capsule) per crop	–0.1	1.2	1.6	1.7
			1.7	2.1	1.8
5	controls	–0.4	–0.5	–0.1	0.1
			0.4	–0.6	0.9

* Unit = modified Horsfall unit.
** Air contacts = units 1, 2, and 3 connected in series for ventilation.

Uninoculated, unexposed controls (unit 5) and air contact controls (units 2 and 3) remained serologically negative throughout the trial. Birds exposed by crop and drinking water (unit 1) and double capsule (unit 4) were all immune by 4 weeks postinoculation.

Experiment 4. Thirty 8-month-old hens were used to study further the syndrome of an AE infection. Data were wanted on the period and extent of egg transmission, contact transmission among chicks, serological response, the relationship, if any, of transmission to egg production and hatchability, occurrence and duration of histological lesions, and elimination of virus in feces.

The flock from which the hens were obtained was considered susceptible on the basis of embryo susceptibility and negative SN tests prior to inoculation. Eighteen of these birds (Group A) were placed in individual compartments within a wire laying battery. Two males from the same flock were kept in a separate cage, but in the same isolation unit, to supply semen. The hens were artificially inseminated throughout the course of the experiment. An additional twelve hens

(Group B) from the same flock were placed on the floor of a colony house and were mated with two males known to be immune to AE. The birds remaining in the original flock were considered isolated controls (Group C).

Three liters and 2 liters of inoculum were administered as drinking water to Group A and Group B birds respectively. Inoculum consisted of the following:

Isolate	Dilution	Quantity
28	1:5	5 ml
1143	1:5	5 ml
774	1:5	16 ml
VR	Undiluted	48 ml
broth	—	500 ml
distilled water	—	4500 ml

Blood samples for SN tests were collected from all birds prior to inoculation and again at 3 and 4 weeks postinoculation. Eggs collected from the 29th through the 35th day postinoculation were tested for embryo susceptibility to AE virus.

At weekly intervals from the 3rd through the 8th week, two birds from Group A were sacrificed and tissues were saved for histological examination. Tissues from two isolated control birds were examined in parallel through the 6th week. Feces for virus assay were collected and pooled twice weekly from six Group A birds.

Eggs and subsequent chicks from Group A birds were individually identified according to hen and date laid. Group B eggs (from hens not trapped) were identified by date only. All eggs from Groups A and B plus 3 dozen from Group C were set weekly for 4 successive weeks. Eggs were candled at 5, 14, and 17 days of incubation and were hatched in the same incubator. On the 22nd day the chicks were taken out and wingbanded. The identity of embryos which had pipped or were dead-in-shell (D/S) was recorded. Brains were harvested from all pipped and D/S embryos in the second hatch only for virus isolation attempts.

Chicks from all three groups within each hatch were held together in a wire battery. Chicks from the first and fourth hatches were held in separate isolation rooms while chicks from the second and third hatches were kept in separate bat-

teries within another room. Since lesions of AE are not easily detected during the 1st week of age isolation attempts were done on brains harvested from all chicks developing clinical signs during the first 8 days. Histological examinations were made on those affected after 8 days of age.

Egg transmission and contact transmission. When noninfected, susceptible controls in the first three hatches were placed together with infected chicks, they developed clinical signs of AE as a result of apparent contact transmission as early as 11 days of age. For this reason, only those chicks affected prior to this age were considered infected by egg transmission. No chicks from the fourth hatch (eggs laid 21 to 28 days postinoculation) were infected either by egg or contact transmission. Complete data of egg production, hatchability, egg transmission, and contact transmission are found in table 4. These results are based on eggs from 16 hens from Group A (two were sectioned at 3 weeks and not included in transmission data), and 12 Group B hens. Data from Groups A and B are presented together because no significant differences were observed. Egg transmission occurred in chicks hatched from eggs laid on the 6th through the 13th days postinoculation. A total of 21 chicks were infected and developed clinical signs of AE at the following ages: 1 day—four chicks, 2 days—two chicks, 3 days—three chicks, 5 days—four chicks, 6 days—five chicks, 7 days—three chicks. Clinical signs due to contact transmission first appeared at the 11th day of age and continued to appear until the 16th day of age in chicks from both the isolated controls and the infected hens. In the latter group, it is only presumed that chicks that developed signs during this time interval were infected as a result of contact. In the first hatch (eggs laid 1 to 7 days postinoculation), all chicks not infected by egg transmission, both from isolated controls and from inoculated hens, were infected by contact. From the second hatch (8 to 14 days postinoculation) 24 of 27 control chicks developed AE from contact whereas only 21 of 44 chicks from the inoculated hens succumbed. There was no egg transmission in the third hatch (15 to 21 days postinoculation of parent

TABLE IV

Egg production, hatchability, egg transmission, and contact transmission in offspring chicks following oral administration of AE virus to 23 susceptible 8-month-old hens. Experiment 4.

	Days Postinoculation																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Number of eggs	19	18	16	17	21	15	16	17	15	11	15	11	17	12	16	17	20	17	21	20	24
Live 18 da. emb.	13	16	14	15	19	14	15	15	13	11	13	8	16	10	15	16	16	16	18	18	22
Pipped or D/S*	3	3	1	2	5	3	6	8	5	5	4	1	2	1	2	1	1	3		2	1
Egg trans.**						2	4	3	3	4	2	1	2								
Contact trans.	10	13	13	13	14	9	5	4	5	2	4	2	4	2					1	1	2
Normal 19 days											3	4	8	7	13	15	15	13	17	15	19

* Dead-in-shell embryos
** Number of chicks infected by egg transmission, contact transmission, or normal at 19 days.

stock), so contact transmission probably occurred because of the close proximity of chicks from the second hatch. Only 10 of 27 control chicks and six of 113 chicks from inoculated hens developed clinical signs of AE from contact transmission in the third hatch.

Of the 16 hens in Group A, 12 had developed virus-neutralizing antibodies by 28 days after inoculation, hence it was known that a definite infection had been established in these particular birds. Examination of the results from these 12 birds (table 5) further clarifies the periodicity of contact transmission. All chicks from eggs laid the 1st through the 8th day postinoculation that did not develop AE from egg transmission became infected by contact. All chicks from eggs laid on the 9th and 10th days postinoculation developed AE as a result of egg transmission. Chicks from eggs laid subsequent to the 10th day postinoculation that were not infected by egg transmission survived, whereas some both from isolated control hens and from the four Group A hens susceptible at 28 days did continue to show evidence of contact transmission. Histological examinations of tissues of surviving chicks from infected (immune at 28 days), non-infected (susceptible at 28 days), and isolated control hens were done. No lesions were found in 39 chicks from the first group but lesions of AE were present in chicks from the latter two groups (6/8 and 7/13 respectively).

Egg production and hatchability. The data presented in table 4 indicates both a drop in egg production and an increased number of pipped and dead-in-shell embryos from eggs laid during the period of egg transmission. Production of Groups A and B combined averaged 65 percent for the 1st five days, 52 percent for the 6th through 13th days, and 66 percent for the 14th through 21st day postinoculation. The corresponding figures for the 12 birds known to be infected in Group A were 72, 50, and 79 percent respectively. Hatchability, based on embryos alive at 18 days, amounted to 68 percent for eggs laid on the 6th through the 13th day postinoculation as compared to 82 percent for 1st through 5th and 84 percent for 14th through 21st day eggs. Hatch-

TABLE V

Egg infection, egg transmission, and contact transmission in offspring chicks following oral administration of AE virus to 8-month-old-hens. Experiment 4.

	Days Postinoculation																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Hen #1			C	C	P			D+	D+		*E*+		N–	N–	N–	N–	N–	P		N–	N–
2	C		C	C		C	D	D+			P+	N–	*E*+	N–	N		N	N	N	N	N
4	C	C		C	D											N	N		N		N
5		C									D+							N			
7																	N	N	N	N	N
8		C	C	D		C	D	D+	P+				D+	N–	P	N	N		N	N	P
9	C		C	C	D		D		D+					N	N		N	N	N		N
12	C	C		C	D	D		D+					N–	N–	N	N		N	N	N	N
15	P		C	C	C	C	*E*+		P+	*E*+	N–	N–	N–	N–		P	N–	N–	N–	N–	N–
16															N	N		P	N	N	N
17	C	C	C	C	C	C	*E*+	C	*E*+		N–	N–	N–		N–	N–	N–		N–	N–	N–
18			C	C	C	*E*+		*E*+		*E*+			*E*+		N–	N–	N–	N–	N–	N–	N–
Live 18 da. emb.	6	5	7	9	7	6	5	6	5	2	5	3	7	6	8	8	9	9	10	9	11
Pipped or D/S*	1			1	4	1	3	4	4		2		1		1	1		2			1
Egg trans**						1	2	1	1	2	1		2								
Contact trans.	5	5	7	8	3	4		1													
Normal 19 days											2	3	4	6	7	7	9	7	10	9	10

* Dead-in-shell embryos

** Egg transmission, contact transmission, normal 19 days — number of chicks.

C = Chick infected by contact transmission, *E* = chick infected by egg transmission, (+ = pos. virus isol.), P = Pipped embryo (+ = pos. virus isol.), D = dead-in-shell embryo (+ = pos. virus isol.), N = chick normal at 19 days (– = negative histology).

ability of eggs from isolated control hens in the 1st 3 hatches was 97, 90, and 93 percent respectively.

Immune response. Preinoculation serum-neutralization tests done on the 30 hens (Groups A and B) were negative. The geometric mean titer (GMT) was .1 with a range of –.6 to .7 for individual neutralization indices (NI). At 3 weeks postinoculation, 18 of 28 hens tested were immune, the GMT of the immune samples being 1.5 and the NI range 1.1 to 2.2. By 4 weeks 22 of 28 birds were positive, raising the immune GMT to 1.7. The NI range remained 1.1 to 2.2.

Embryo susceptibility tests done on eggs collected prior to inoculation indicated no immunity (100 percent susceptible). Weekly samples of about 24 eggs from the isolated control hens remained fully susceptible throughout the experiment. Of 128 eggs collected from the inoculated hens of Groups A and B between the 29th and 35th days postinoculation, only 19 (15 percent) were fully susceptible. All susceptible eggs from the Group A hens (14 of 77) were laid by 3 birds having negative SN tests at 4 weeks postinoculation.

Histopathological responses. Microscopic lesions of AE were found in tissues from one of two hens at 3 weeks and two of two hens at 4, 5, and 6 weeks postinoculation. One of two Group A birds examined at 7 weeks was positive while both birds done on the 8th week were negative histologically. Two isolated controls examined at 3, 4, 5, and 6 weeks were negative. The lesions observed in infected hens were indistinguishable from those found in natural outbreaks of AE in breeders. In two instances within the past year, AE was diagnosed by the use of histopathology in flocks from which birds had been submitted to the laboratory. Both flocks were undergoing a slump in production at the time birds were examined. The diagnoses of AE were later confirmed by the appearance of AE in chicks from eggs laid during the drop in production.

Virus-assay of feces. Each feces pool collected from six Group A birds was administered in the drinking water to five 4-day-old susceptible chicks held in Horsfall units as

described previously. Ataxia and paralysis were seen in all chicks in the groups given feces collected at 5, 9, and 12 days postinoculation. These clinical signs of AE were first evident as early as 11 days following administration of the feces. Chicks receiving feces pools collected preinoculation and at 16, 19, 23, 30 and 37 days postinoculation appeared normal throughout a 28-day observation period. Tissues from two chicks in each group were sectioned for histological examination at 21 days after inoculation. Lesions of AE were found in the brains and gizzards of chicks inoculated with feces collected at 5, 9, and 12 days. The remaining three birds in each group were bled for SN tests at 28 days postinoculation. Positive samples were observed in the 5, 9, and 12 day feces pool groups. Data are found in table 6.

TABLE VI

Pathological and serological responses of 4-day-old chicks orally inoculated with feces from 6 adult birds infected with AE

Feces pool (Days postinoc. collected)	Clinical AE	AE lesions*	Serol. response 28 da. postinoc.		
	Total inoc.	No. exam.	Pos.	GMT	(NI range)**
Preinoc.	0/5	0/2	0/3	.1	(0 to .3)
5 da.	5/5	2/2	3/3	2.5	(2.2 to 2.9)
9 da.	5/5	2/2	3/3	2.7	(2.3 to 3.2)
12 da.	5/5	2/2	3/3	2.2	(1.9 to 2.5)
16 da.	0/5	0/2	0/3	0	(0 to .1)
19 da.	0/5	0/2	0/3	.3	(0 to .7)
23 da.	0/5	ND	0/3	.1	(0 to .3)
30 da.	0/5	ND	0/3	.3	(0 to .7)
37 da.	0/5	ND	0/3	.1	(0 to .3)
Uninoc Controls	0/6	ND	0/4	.3	(0 to .7)

* Lesions in brain, gizzard and proventriculus of chicks sacrificed 21 days postinoculation. ND = not done.

** Pos. = neutralization index (NI) of 1.1 or greater. GMT = geometric mean titer.

These results are contrary to those obtained in previous work using only the VR strain as inoculum. This strain was not demonstrated in feces collected over a period of 21 days following oral inoculation of birds. The birds, however, were serologically immune at 28 days postinoculation.

Virus isolation attempts. Supernates of 1:10 brain suspensions prepared from all chicks infected by egg transmission

and from Group A and Group C pipped and dead-in-shell embryos in the second hatch were injected intracerebrally into susceptibile day-old chicks. Brains from the 21 egg-infected chicks were all positive. Of 13 brain suspensions from Group A pipped and D/S embryos, 12 were positive for AE virus. The one negative sample was from an egg laid by a hen serologically negative at 28 days. Two brain suspensions from D/S Group C (isolated control) embryos also yielded negative results on isolation attempts.

Experiment 5. Previous work here had indicated that AE isolate 1288, when injected into the yolk-sacs of 6-day embryos, did not markedly reduce hatchability but did cause most of the subsequent chicks to develop clinical signs of AE within the 1st week after hatching. Because of these characteristics, it was employed to provide a source of infected chicks to further study contact transmission. Sixty susceptible 6-day-embryos were inoculated via the yolk-sac with this isolate. Incubated with these were 112 uninoculated control eggs. On the 18th day of incubation, 57 surviving eggs from the inoculated group and 56 control eggs were placed in separate trays within the same incubator (A) for hatching. The remaining 56 control eggs were hatched in a separate incubator (B). Chicks were taken out on the 22nd day of incubation. In incubator B, 55 control eggs hatched and one pipped. Control eggs in incubator A hatched equally well giving 55 chicks and one dead-in-shell embryo. Of the 57 inoculated eggs in incubator A, three were dead-in-shell, two pipped, and 52 hatched. Chicks were wing-banded and distributed to wire batteries in the following manner:

Group	Unit 1	Unit 2	Unit 3
Infected - incubator A	52		
Controls - incubator A	30	25	
Controls - incubator B	28		27

Observations of the chicks were made daily. Clinical signs were recorded at first appearance though most affected chicks were left with their respective groups until ataxia and paralysis had become quite pronounced. On the 5th through the 10th day after hatching, one or two chicks from each of the groups were sacrificed and tissues were removed for

histological examination. From the 11th day on, only occasional ataxic chicks were sectioned in order to verify the diagnosis of AE. Tissues from all survivors at 22 days of age were examined histologically.

Clinical AE in chicks hatched from the inoculated eggs first appeared at one day of age. Additional chicks became affected through the 6th day of age making a total of 49 of 52 chicks having signs. Chicks in all other groups exposed (either in the incubator, battery, or both) to the infected group developed first signs of ataxia and paralysis at 10 days of age. Affected chicks from these groups continued to appear through the 17th day. Data on the appearance of clinical signs are found in table 7. Discounting those chicks removed for sectioning prior to the appearance of clinical signs, 22 of 24 (92 percent) chicks hatched and raised with the inoculated group developed clinical AE as compared to 49 of 52 (94 percent) affected in the latter group. Chicks hatched with the inoculated group but raised separately succumbed to the extent of 15 out of 18 (83 percent). Those hatched separately but raised with the inoculated group were affected at a rate of 16 of 21 (76 percent). Control chicks hatched and raised separately developed no clinical signs of AE throughout the trial.

TABLE VII

Contact transmission when susceptible chicks are exposed to infected chicks during hatching, raising, or both. Experiment 5.

Group	Signs AE No. chicks	Days age 1st Signs	Lesions* survivors
Inoculated**	49/52 (94%)	1- 6	1/3
Control 1 (hatched and raised with inoculated)	22/24 (92%)	10-16	1/2
Control 2 (hatched with inoculated; raised separately)	15/18 (83%)	10-16	3/3
Control 3 (hatched separately; raised with inoculated)	16/21 (76%)	10-16	3/5
Control 4 (hatched and raised separately)	0/19	—	0/6

* Histological lesions of AE/chicks surviving at 22 days of age.
** Inoculated = AE virus inoculated into yolk-sacs of 5-day embryos, embryos allowed to hatch.

The question arose as to whether or not the high incidence of clinical signs following contact was a result of infection through the wound produced by wing-banding rather than through more natural routes. That the wound was not necessary was proven when 19 egg-infected and 21 contact controls were hatched and raised together without banding. Results obtained were essentially the same as in banded birds.

Histological lesions of AE were observed in the gizzard of egg-infected chicks as early as 5 days of age, and in brain and proventriculus at 9 days of age. Gizzard and proventriculus lesions appeared at 8 days and brain lesions at 10 days in control chicks hatched with the infected ones. Chicks hatched separately but raised with the infected group had lesions in the gizzard and proventriculus at 10 days and brain lesions at 12 days of age. No lesions were found in chicks hatched and raised separately.

DISCUSSION

The results presented here offer evidence strongly suggestive that AE is an enteric infection in growing and adult birds. Of paramount importance were two observations: (1) oral administration of virus induced a syndrome indistinguishable from that seen in naturally occurring outbreaks of AE, and (2) virus was eliminated in the feces of infected birds over a period of several days and at a level sufficient to infect young susceptible chicks by the oral route. Although one could not conclude that ingestion of virus is the only mode of infection, it undoubtedly plays a very important role. Certainly, the possibility of infection via the respiratory tract, particularly in young chicks, should not be excluded. Birds placed in a sterile battery within an AE contaminated colony house became infected but to a much lesser extent (40 vs. 85 percent) and some time later (7 vs. 3 weeks) than birds placed on the floor within the same house. Since the litter was reasonably dry and dusty it is conceivable that infection in the battery-held birds occurred either as a result of their feed and water being contaminated or by direct inhalation of virus from the litter and feces.

Young chicks inoculated via the crop by a double capsule technique, thus avoiding contamination of the upper respiratory tract, developed positive serology within 28 days. Other chicks held in Horsfall units and exposed to air previously passed through a unit holding orally infected birds failed to become immune over an 8 week period. These findings lend further support to the conclusion that infection may normally take place through the intestinal tract.

Virus was detected in the feces of orally inoculated adult birds over a period of several days. The minimum time limits on elimination of virus were the 5th through the 12th days postinoculation. Feces, collected on the 5th, 9th, and 12th day postinoculation produced clinical signs of AE in 4-day-old chicks when 0.2 gram per bird was administered orally. Although the actual quantity of virus excreted by infected birds was not determined, it appears that there was a sufficient amount to account for the source and spread of infection to susceptible birds.

In each of two experiments, oral administration of AE virus to susceptible adult breeders induced egg transmission. Observations of egg transmission in the 1st experiment served primarily to establish that the hens had undergone an outbreak of AE similar to field cases. In the second trial (experiment 4), information regarding the nature of the infection was obtained. It was found that a drop in egg production, decreased hatchability, and egg transmission of the virus occurred essentially concurrently and between the 6th and 13th days postinoculation. As mentioned previously, virus was shed in the feces during the same interval. Histological lesions of AE and positive serology were evident as early as 21 days postinoculation. The results on production, hatchability and egg transmission are in agreement with the observations made by Taylor, *et al.*[10]

Of particular interest was the occurrence of clinical AE in susceptible chicks held in contact with those infected by egg transmission (experiment 4). Since this was wholly unexpected when the experiment was designed, no chicks from the isolated control hens were hatched and raised in isolation.

The fact that the control hens remained susceptible throughout the experiment, however, negates the possibility of egg transmission as the cause of clinical signs in these chicks. Clinical AE developing as a result of contact transmission consistently occurred first on the 11th day after hatching in each of three hatches. Chicks did not become affected after the 16th day of age. The percentage of contact transmission in control chicks decreased progressively in the first three hatches (100, 89, and 37 percent respectively), probably due to less exposure in the latter hatches. As there was no egg transmission in the third hatch, these chicks received their only exposure from an adjoining battery which held second hatch chicks. Contact transmission in chicks from infected hens decreased at a higher rate than in control chicks and ceased entirely after the period of egg transmission (13th day postinoculation). The lack of susceptibility of chicks coincident with the cessation of egg transmission by the dams suggests the appearance of circulating antibodies in the dams. Evidence of parental immunity was observed in some chicks from eggs laid as early as 11 days after inoculation.

The very high percentages of chicks that became afflicted with AE in some hatches, and certainly the 100 percent loss in the first hatch are not in accord with observations on most field cases. In the latter, losses of only 5 to 10 percent are common though they may reach above 50 percent. As noted previously, the majority of breeder flocks are immune to AE. Most hatcheries depend upon eggs from many sources for hatching purposes. In doing so, the chances of diluting susceptible chicks with parentally immune chicks are increased, thus decreasing the possibility of contact transmission occurring at a high overall rate. It would seem advisable that eggs from known susceptible flocks be hatched alone or with eggs from known immune sources.

The ease with which susceptible chicks may become infected was quite apparent from the results of experiment 5. Chicks infected by experimental egg transmission provided adequate virus to infect a large percentage of susceptible chicks either within the incubator during hatching, or in wire

batteries after hatching. In these instances, clinical signs from contact transmission first appeared at 10 days of age as compared to 11 days for each of three hatches in experiment 4. It is interesting to note that the minimum incubation period following oral administration of virus-contaminated feces was also 11 days. Egg transmission, on the other hand, either natural (experiment 4) or experimental (experiment 5) produced clinical signs as early as 1 day of age and not later than 7 days of age. Generally, there was a lapse of about 4 days between the end of egg transmission and the beginning of contact transmission in which no chicks became affected. It is possible that the majority of chicks afflicted in field cases are infected as a result of contact with a few egg-infected chicks. Furthermore, the possibility that chicks hatched from susceptible dams may become infected from AE contaminated premises deserves attention.

It is evident that the manifestation of clinical AE following contact exposure must in part depend upon the age of the chicks and the degree of exposure. The age at which chicks can become infected but yet not have signs of disease needs to be determined.

There were no observed adverse effects other than a drop in egg production upon the hens following oral administration of AE virus. Egg transmission occurred for a relatively short period of time and immunity was established within 2 to 3 weeks following inoculation. This information suggests that vaccination of susceptible breeding flocks may be of a practical nature. By using the embryo susceptibility test, the immunity status of flocks could be known shortly after egg production commences. If a susceptible flock was vaccinated and the eggs eliminated for hatching purposes for a short time, the danger of a future outbreak at an unknown time would be removed. Certainly an approach such as this warrants further investigation.

Perhaps of even greater interest is the potential application of findings to the development of an effective immunization program for growing birds. Oral vaccination of young birds (after the disappearance of parental antibodies and

prior to egg production) would have several obvious advantages. Application would be simple and would eliminate the need for handling each bird as is the case with wing-web or inactivated vaccines. The disease would resemble that occurring naturally and should therefore be inapparent. Also, the birds would be immune prior to the commencing of egg production. It is conceivable that only a small percentage of birds within a flock dosed with vaccine could adequately spread the infection to others.

In the development of such a program, selection of a suitable strain of virus would be important. Previous work[2] indicated that a very high oral dosage of VR strain virus was required to stimulate an immune response. Furthermore, contact controls held in the same room with birds inoculated with VR virus remained susceptible for several weeks. Other experiments indicated that this strain was not recoverable from the feces of orally inoculated birds over a period of 21 days, even though the birds were serologically immune at 28 days. Results reported in this paper were considerably different. Contact transmission occurred within a short period of time and to a large percentage of the contact controls. Also, feces were demonstrated to contain viable and infectious AE virus. These differences were undoubtedly due to varied characteristics among the isolates used. For the experiments reported here, inocula invariably contained one or more isolates of rather recent origin that had undergone few if any passages in laboratory hosts. The VR strain, on the other hand, had been passaged intracerebrally in chicks a total of 150 times and had an additional 36 passages in embryonating eggs. Although this strain had retained neuropathic properties, it would appear that enterotropic tendencies, if originally present, were lost by passage. Isolates less removed from their original hosts appeared to be both neuro- and enterotropic.

The selection of an isolate that is relatively non-pathogenic for the central nervous system, is easily propagated within and excreted from the intestinal tract, and suitably immunogenic should provide a safe and effective vaccine virus for oral administration.

SUMMARY

Studies on the epizootiology of avian encephalomyelitis (AE) resulted in the following findings:

1. Susceptible birds placed in a colony house previously holding AE infected chickens developed positive serology within 3 to 7 weeks.

2. Chicks could be infected by the intestinal route to the exclusion of the respiratory tract.

3. Oral (drinking water) administration of AE virus to susceptible adult breeders produced a syndrome indistinguishable from that observed in field outbreaks. A drop in egg production, lowered hatchability, and egg transmission of virus occurred between 6 and 13 days postinoculation. Virus was demonstrated in the feces during the same interval.

4. Transmission of AE took place from egg-infected chicks to susceptible contact chicks following exposure either within the incubator during hatching or in batteries following hatching. Clinical signs appeared at about 11-16 days following contact.

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TRANSMISSION OF CHRONIC RESPIRATORY DISEASE IN CHICKENS*

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CHRONIC respiratory disease (CRD) is recognized as one of the important poultry disease problems confronting the industry at the present time.[2] Especially is this true for the broiler industry. It has been generally accepted that the cause of CRD is the avian pleuropneumonialike organism (PPLO).[1,4,8,9] Considerable knowledge concerning this disease has been gained during the past decade, although it should be recognized that only very limited information has been established relative to the transmission of the disease. The sources of the infection and the channels through which it may spread need further elucidation.

Egg-borne infection is believed to be the most common source of dissemination from one generation to the next.[3,5,6,10] This mode of transmission may express itself in many different patterns that may be influenced by certain factors. Investigations concerning the elimination of the organism in the egg have revealed that the rate and persistency of the shedding of the agent may be extremely variable.

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While the rate and persistency of spread may vary in different flocks or even within the same flock, it is observed that as the progeny attain sexual maturity, approximately 100 percent of the birds in the flock will become serologically positive.

The source and mode of spread of the infection play an important role in the control and eradication of this disease. In the investigations reported in this paper, consideration has been given to the degree and severity of the infection in a breeding flock and to their possible influence on the dissemination of the agent via the egg or through direct and indirect contact between the infected and noninfected chickens.

MATERIALS AND METHODS

Studies were conducted in four CRD-positive chicken flocks dealing with egg and contact transmission of the avian pathogenic Mycoplasma organism.

Experiment 1. This experiment was designed to study incubator and brooder transmission of the avian PPLO. The source of positive eggs was a flock comprised of Barred Plymouth Rock (BR) females and Rhode Island Red (RIR) males, designated as flock I, that had experienced a severe CRD outbreak. The birds revealed a marked decline in egg production, poor egg quality, and 50 percent hatchability of chicks during the height of the disease. A high percentage of the cull chicks and embryos from nonpipped and pipped eggs exhibited lesions for several months following the disease outbreak. The Sex Linked (SL) progeny, obtained from this flock at the height of the disease outbreak and reared on a commercial farm, manifested CRD at 3 days of age and a 3 percent mortality at 6 days of age.

The control eggs were obtained from flock II, an established CRD-free RIR flock maintained at the University Poultry Farm.

Hatching eggs for this study were obtained approximately 5 weeks after the disease occurred in the positive flock. Fertile eggs from the positive and negative flocks were hatched in a single incubator to determine if transmission may occur

in the hatcher. Negative control chicks, hatched in an isolated incubator, were reared with progeny from the positive flock to detect evidence of transmission by contact during the brooding period. A portion of the chicks from the two sources were stressed at 5 weeks of age with a virulent strain of infectious bronchitis virus (IBV) in an attempt to precipitate the CRD syndrome. Serology was the main criterion for determination of transmission. Serologic determinations were made with the rapid-serum-plate agglutination test. The Adler S6 strain was used in preparation of the antigen by this laboratory. Biweekly or monthly tests were conducted on the RIR and SL birds for 6 and 12 months, respectively.

Experiment 2. This was a study of egg transmission of CRD in flock III comprised of two age groups of BR birds, pullets and yearling hens, that had undergone a natural outbreak of CRD at different time periods. The stock for both groups of birds was obtained as baby chicks from the same breeder-hatcher, the yearling group in one hatching season, and the pullet group in the following season, 12 months later. The hatchery has had a long CRD history.

The pullet flock was enrolled in a laboratory vaccination program for Newcastle disease (ND) and IB that included periodic serologic tests for CRD. At 3 months of age, this group was positive for CRD. As part of the vaccination program, susceptible CRD-free birds were added to the flock 6 weeks following the second viral vaccination at 4½ months of age. Evidence of CRD was detected among these susceptible control birds within a month after placement in the flock.

The yearling flock had also been enrolled in a laboratory vaccination program for ND and IB, and random tests for CRD indicated a negative status. The entire flock, at one year of age, was tested for CRD and found negative by the rapid-serum-plate test. A portion of these birds was retained for production for a second year. The birds were held in two pens in the laying house with the pullets. Respiratory signs, a slight mortality, a slight decline in egg production, and poor egg quality, were observed in these yearling hens approximately 2 months following the placement of the susceptible

controls in the two pullet pens. The yearling hens were separated from the pullets by a feed room. The disease was diagnosed by isolation of the agent in embryonated eggs and birds, and confirmed by preclinical and postclinical serologic tests. No signs were apparent in any of the pullets and in only a few of the susceptible control birds that had been placed in the two pullet pens.

Eggs for this study were obtained from both groups of birds, approximately 3 weeks after the onset of the disease among the yearling stock and 24 weeks after the onset of the disease in the pullets. The eggs were incubated and hatched in separate incubators. The chicks from each group were reared in isolated quarters. Both groups of progeny were stressed by exposure to IBV at 4 weeks of age to precipitate the CRD syndrome. The birds were necropsied at 8 weeks of age. The main criteria for determination of transmission were: clinical signs, serology, and gross pathology of the birds.

Experiment 3. In this experiment egg and contact transmission were studied. The positive flock (flock IV) was comprised of BR females and RIR males and had experienced a natural outbreak of CRD. The supply hatchery was the same as described for the pullets used in experiment 2. The birds became serologically positive for CRD by the time they were 4 months of age. The hatching eggs for this study were obtained 4 months later.

The control eggs were obtained from a CRD-free White Plymouth Rock (WR) flock (flock V).

The chicks from both groups were hatched and reared in separate isolated quarters. At 5 weeks of age, the chicks were moved to a poultry house and at 7 weeks of age a portion of the chicks from each group were stressed by ND vaccination. The SL males and all the WR birds were sacrificed at 11 weeks of age and examined for gross evidence of CRD. The SL pullets were raised to maturity and tested at periodic intervals for serologic evidence of CRD.

The main criteria for determination of CRD transmission were: clinical signs, serology, and gross pathology.

Experiment 4. This experiment was designed to study hatcher transmission of CRD. The yearlings and pullets of flock III, described in experiment 2, provided the positive source of hatching eggs. A time interval of 6 weeks elapsed between the two experiments. The general condition of the yearling flock had improved considerably.

The control eggs were obtained from flock VI, a White Leghorn (WL) flock with no history of CRD.

Hatching eggs from the yearling flock and control groups were hatched in a single incubator. At the time of hatching, the chicks were divided according to original source and reared in isolated quarters for 4 weeks. At this time, a portion of each group was stressed with IBV in an attempt to precipitate the CRD syndrome. The birds were necropsied at 8 weeks of age.

The pullet eggs were hatched in a separate incubator and the progeny were examined at one day of age.

The criteria for evaluation of transmission were: clinical signs, serology, and gross pathology.

Experiment 5. This experiment, extending over a period of 12 months, was conducted to study contact transmission of CRD among mature chickens in an environment that simulated commercial conditions. The positive chickens were obtained from a BR flock, designated as flock VII, that was positive serologically and manifested only slight clinical evidence of the disease. The negative birds were obtained from the CRD-free flocks (RIR, WR, WL).

Two adjacent pens, D and E, were established. Pen D was comprised of 50 BR positive hens, 25 RIR susceptible hens, 7 RIR susceptible males, 17 RIR susceptible pullets, and 7 WR susceptible pullets. Pen E was comprised of 50 CRD-positive BR hens, 53 WL susceptible hens, 4 RIR susceptible males, and 4 WR susceptible males. The birds in each pen were tested serologically at weekly or biweekly intervals.

The first week of the experiment all birds in pen E were cultured for PPLO. Tracheal swabbings were made in beef heart infusion broth to which was added 10 percent horse serum, penicillin (1,000 units per ml), and thallium acetate

1:4000 concentration). The cultures were checked for colonial appearance on the corresponding agar medium that was enriched with 10 percent horse serum. The phenol red medium described by Grumbles *et al.*[7] was used to check carbohydrate reactions. This medium was also enriched by 10 percent horse serum without inhibitors. All cultures were also checked for hemagglutination activity. Tracheal swabbings were also taken from a portion of these birds at 12 months, the termination of the experiment.

The eggs collected over an intermittent period of 17 days during the 4th and 6th month of the experiment were cultured for PPLO. In general, the whole yolk was cultured for PPLO in media similar to that described for the tracheal swabbings. All other eggs obtained from this pen through the 7th month were hatched and the embryos and chicks were examined for gross evidence of CRD. The settings were made approximately at weekly intervals. Two of these latter settings were pedigree hatched.

At the 5½ month period, 32 BR chickens were removed from pen D and challenged with a virulent CRD strain. The inoculum was strain Hy (S6 type) of *M. gallisepticum* that was isolated at this laboratory. At 8 months, 32 WL and 32 BR chickens were removed from pen E for similar resistance studies.

At 6 months, 40 susceptible, 6-week-old WR chicks were placed in pen D with the CRD serologically positive birds, to determine if CRD would be transmitted to these birds. One-half of the birds were placed directly in the pen and the other half were placed in a battery raised slightly above the floor level. All the birds were bled at biweekly intervals for 3 months. At this time the birds were sacrificed and examined for evidence of CRD. The nasal passages and tracheas were also cultured for PPLO.

The criteria for evaluation of transmission among these birds were: clinical signs, serology, cultural tests, and gross pathology.

Experiment 6. This was a study concerning CRD transmission in a commercial breeding flock. Two groups of WR

birds, designated A and B, located on a commercial poultry farm, were tested at periodic intervals for serologic evidence of CRD infection. Group A was comprised of 3500 chickens that were obtained at 9 to 12 weeks of age from a breeder-hatcher that has been actively engaged in a CRD control program for several years. These birds had been brooded and reared on 6 small poultry farms associated with the hatchery.

The birds in all groups were vaccinated for ND, IB, and fowl pox according to laboratory recommendations.

The birds were first tested for CRD at 8 to 12 weeks of age while still on the premises where they were reared. Subsequent tests were conducted on the premises where the birds were reared to sexual maturity and maintained for breeding.

At 10 months of age, 476 birds of certain breeding qualities were selected from this flock to supply hatching eggs for replacements for the following year. These replacements were hatched and brooded on the same farm that housed the breeders. The remainder of the breeding flock supplied hatching eggs to the hatchery. At periodic intervals, cull chicks and pipped eggs were submitted to the laboratory for examination for evidence of CRD infection. Progeny from group A that remained on the farm have been tested serologically for CRD for a period of 12 months.

Group B, approximately 800 birds, was derived from the flock owner's stock that was recognized as CRD negative the previous year. This group was tested by the serum-plate test at periodic intervals for 12 months for evidence of CRD. A limited number of cull chicks and pipped eggs were examined from this group since this strain was to be discarded after completion of its laying year.

Experiment 7. The natural spread of CRD was investigated in 8 commercial flocks that were vaccinated with combination ND and IB vaccines (water or dust) at 1 to 1½ months of age and revaccinated at 4 to 4½ months of age. The stock in all flocks originated from positive sources. Susceptible, CRD-free, mature chickens were placed in contact with the vaccinated birds 1½ to 4 months after the last

vaccination. Spread of CRD infection among the vaccinated and control birds was determined by periodic rapid-serum-plate tests. Random tests were made prior to, and after the first and second vaccination, and then at approximately monthly intervals until the birds were one year of age. The susceptible controls were tested at biweekly and monthly intervals.

RESULTS

Experiment 1. In this experiment, 360 eggs from positive flock I and 285 eggs from negative flock II were used. A designated number of fertile eggs from each flock were hatched in a single incubator to determine if transmission may take place from the positive to the negative stock. These chicks were divided at the time of hatching according to flock origin and each lot was reared in isolated quarters. The remaining fertile eggs from the negative flock were hatched in a control incubator, and at the time of hatching a portion of these chicks were removed to isolated quarters and reared with an equal number of chicks from the positive hens to determine if transmission may occur through contact in the brooder. The remaining chicks from the negative birds were reared in isolation. Certain groups were stressed at 5 weeks of age by IBV inoculation. The birds were reared to maturity on nonpoultry farms. Results are presented in table 1.

Eight embryos from pipped and nonpipped eggs and one day-old chick obtained from the positive flock revealed CRD lesions and the causative agent was isolated in embryonated eggs and artificial media. However, no further evidence of transmission was detected by serology in any of the progeny, the control progeny having been tested for 6 months and the positive flock progeny for approximately 12 months.

Experiment 2. In this trial, 360 eggs from the yearling hens and 144 eggs from the pullets of flock III were used. A designated number of chicks from each group were reared in strict isolation. The remaining chicks and embryos were examined for gross CRD lesions. At 4 weeks of age, the chicks were stressed with IBV and observed for an additional 4-week period. Results are given in table 2.

TABLE I

Incubator and brooder transmission of CRD in progeny from positive flock I and negative flock II. (Experiment 1)

Flocks	CRD status	Settings	Fertile eggs (No.)	Incidence of lesions: Embryos No.	Embryos No.+	Day-old chicks No.	Day-old chicks No.+	Observations in reared chicks: No.	Distribution in isolation rooms I	II	III	IV	CRD Findings
I	+	A_1	240	12	5	77	1	151	50			101*	Negative
		B	79	10	3	69	0	0					
II	—	C_1	100	2	0	18	0	80			80*		Negative
		D	165	12	0	28	0	125	50*	75			Negative

1 Groups hatched in same incubator.
* Challenged at 5 weeks with virulent bronchitis strain.

TABLE II

Transovarian transmission of CRD in positive flock III (Experiment 2)

Groups*	Incidence of CRD lesions: Embryos No.	Embryos No.+	Day-old chicks No.	Day-old chicks No.+	Chicks reared No.	Number of chicks positive: Signs	Lesions	Serology
Pullets	9	0	25	1(?)	102(a)	0	0	0
Hens	67	22	154	3	102(b)	49	97	97

* The hatching eggs were obtained 24 weeks after CRD onset in pullets and 3 weeks after onset in hens.
(a) Eight birds died prior to termination of experiment.
(b) Four birds died prior to termination of experiment.

Lesions of CRD were observed in 22 embryos and 3 day-old chicks that were obtained from the yearling hens. Four chicks from the reared yearling group died prior to stressing. One of these chicks manifested gross CRD lesions at 6 days of age. At the termination of the experiment at 8 weeks, the following pathology was observed in the 98 chicks of yearling stock: sinusitis—43 percent, tracheitis—98 percent, and aerosacculitis—31 percent. PPLO was isolated from this group in embryonated eggs and artificial media. No definite evidence of CRD was observed in the pullet progeny.

Experiment 3. In this study, 540 hatching eggs from CRD-positive flock IV and 120 eggs from negative flock V were used. At hatching time a total of 362 SL chicks and 100 WR chicks was selected for rearing. The remaining cull chicks and embryos, 135 from the SL and 20 from the WR group, were examined for CRD lesions. Evidence of CRD was observed in one cull chick obtained from the SL group.

An adventitious outbreak of infectious bronchitis occurred in both groups of chicks at 5 weeks of age. At 7 weeks, 49 WR chicks and 232 SL chicks, placed in separate pens, were stressed with the Roakin ND virus. Newcastle disease also spread to a third pen of 50 WR and 110 SL birds that had not been stressed. At 11 weeks, 111 SL pullets were selected for rearing to maturity from the three pens of birds. The remainder of SL and WR birds were sacrificed and examined for CRD lesions.

No further evidence of CRD was observed in the progeny of the positive or negative flocks. The SL birds that had been reared to maturity remained serologically negative for CRD for 2 years, the time of the last test.

Experiment 4. A total of 360 hatching eggs was obtained from positive flock III: 180 eggs from the pullet group, and 180 eggs from the yearling hens. Also, 108 hatching eggs were obtained from CRD-negative flock VI (WL). The fertile eggs from the yearling group and the negative stock were hatched in the same incubator. The chicks were divided at the time of hatching according to flock origin and reared in isolated quarters. At 4 weeks of age, a portion of the chicks

from each group were stressed by exposure to IBV. Unfortunately, accidental bronchitis infection also appeared in the nonstressed chicks 13 days later.

No lesions were observed among the 8 embryos and 154 day-old chicks examined from the pullet stock or among the 2 embryos and 6 cull chicks from the WL control stock. Definite lesions were observed in two of 15 embryos and one of 80 day-old chicks examined from the yearling stock. Two chicks from the reared-yearling group revealed evidence of CRD prior to vaccination. One chick that died at 5 days of age exhibited aerosacculitis and the other was positive serologically at 4 weeks of age but revealed no lesions.

The progeny of the yearling flock, examined at 8 weeks of age, manifested CRD lesions and positive serology. Results are presented in table 3. The group that was stressed at 4 weeks of age revealed a higher percentage of lesions and positive serology than did the group that became infected with adventitious IB approximately 2 weeks later. The CRD agent was isolated in embryonated eggs, artificial media, and birds. No evidence of CRD was detected in the WL chicks examined at this time.

TABLE III

Incubator transmission of CRD in progeny from positive flock III and negative flock VI (Experiment 4)

			Observations in reared chicks								
			Distribution of chicks at 4 weeks of age in isolation rooms**								
			I			II			III		
Breeding flock	CRD status	No. chicks*	No.	No.+		No.	No.+		No.	No.+	
				L	S		L	S		L	S
III	+	75[1]	34	2	1				36	11	14
VI	—	68[2]	34	0	0	33	0	0			

* The two groups of chicks were hatched in the same incubator
** The birds in Rooms II and III were challenged with potent IBV
[1] Five died before 4 weeks of age (2 positive for CRD)
[2] One died before 4 weeks of age
L - lesions S - serology

Experiment 5. Two BR hens in pen D showed respiratory signs during the course of the experiment, one bird for 1 month and the other for 10 months. Respiratory signs were detected in 3 BR hens in pen E during the first month of the experiment.

The rate of agglutinin production in the susceptible birds has been reported in a previous publication.[9] A portion of the mature susceptible birds in pens D and E revealed evidence of CRD, by a positive serology, within 30 days after initial contact. After 3 months' cohabitation, essentially all the birds were positive. The transmission of the infection occurred with only slight clinical evidence of the disease in a few birds.

Tracheal swabbings taken from the birds in pen E, 7 days after the start of the experiment, yielded the following number of isolations either in artificial media or embryonated eggs: 17 from 53 WL hens and 26 from 50 BR hens cultured. At the termination, the 17 WL hens and 14 BR hens that remained were cultured for PPLO; 7 WL hens yielded the organism.

The results of cultural studies of fresh eggs and examination of progeny obtained from the birds in pen E are presented in table 4. A total of 477 fresh eggs collected during the 4th to 6th month of the experiment, 238 from 42 BR hens and 239 from 37 WL hens in production, was cultured for PPLO. The PPLO was isolated from 22 WL eggs representing six different hens. The number of PPLO isolations over the number of eggs cultured from each of the six hens was as follows: 8/11, 5/10, 1/6, 3/4, 1/1, 4/4. Two of these hens were positive for PPLO on terminal culture of tracheal swabbings; whereas, the first cultures were negative.

The embryos and chicks obtained from 27 settings from each group were examined for evidence of CRD. Twenty hatches were found positive in the WL group. The first positive hatch was set 57 days following the start of the experiment. The lesions produced in the embryos and day-old chicks were extensive. Seven intermittent settings were positive in the BR group. Slight lesions were observed in the BR progeny.

TABLE IV

Chronic respiratory disease transmission studies in mature chickens (Experiment 5)

Items	Groups	
Breeds	Barred Rocks	White Leghorns
CRD exposure	Natural Outbreak	Contact Exposure
No. of birds	50	53
No. eggs positive/no. cultured	0/238(a)	22/239(a)
No. positive hatches/no. hatches	7/27(b)	20/27(c)
No. chicks positive/no. examined	7/1976	43/1784
No. embryos positive/no. examined	1/288	25/235

(a) Fresh eggs collected 4 to 6 mos. post contact.
(b) Positive hatches: 4, 5, 9, 15, 19, 23, 25.
(c) First positive hatch - Hatch 5 (Eggs set 57 days post contact). Negative hatches - 1 to 4, 15, 24, 27.

Two pedigreed settings of 132 WL and 128 BR eggs, collected during the 7th month of the experiment, revealed CRD lesions in one embryo and two day-old chicks, the progeny of two WL hens. One of these hens had also yielded PPLO on culture of fresh eggs at an earlier date.

A portion of the birds in each of the two pens, group D at 5½ months and group E at 8 months, were challenged with a virulent CRD strain to determine the degree of resistance acquired from infection by these birds in comparison with susceptible controls of similar age and breed.

The 32 BR birds from group D and 32 controls were given the CRD agent via the sinus and trachea in four concentrations: undiluted, 10^{-2}, 10^{-3}, and 10^{-4}. The birds were sacrificed at the end of a 3-week observation period. Among the naturally infected birds, only a few manifested questionable signs and lesions of the disease; whereas, all the susceptible birds were definitely positive in all dilutions with the exception of the 10^{-4} dilution in which 7 of 8 birds were positive.

The group E birds, 32 WL and 32 BR, were challenged with undiluted material while the 40 susceptible controls, 20 WL and 20 BR, were inoculated with different dilutions: undiluted and 10^{-3} through 10^{-6}. The response in these birds was similar to those of group D. The end point of infection based on signs and lesions in the controls was in the 10^{-4} and

10^{-5} dilutions. One WL hen that had remained serologically negative for CRD for 8 months in pen E was also found susceptible for CRD on challenge.

Serology was the main criterion for detection of transmission in the 40 WR chicks that had been placed in pen D with the 53 serologically positive mature birds at the 6th month period of the experiment. Negative results were obtained by serology during the 3-month testing period of these birds. However, terminal tests at the time of necropsy revealed that 7 of the 20 birds held in direct contact were positive serologically, 3 were positive for lesions, and 10 birds were positive by cultural tests; a total of 11 different birds. The indirect contacts, held in the battery, were negative serologically and only 4 of 20 were positive by culture. The experiment was terminated before transmission became definitely apparent.

The 53 serologically positive, mature birds from this pen were cultured for PPLO in artificial media and examined for gross lesions of CRD at the termination of this experiment. Thirty-one birds were positive on culture and none for gross lesions of CRD.

Experiment 6. The 3500 birds that comprised group A were tested serologically for CRD for 13 months by the rapid-serum-plate test. A total of 20 doubtful reactors was detected by the first three tests: 4, 8, and 8, respectively. Fifteen of these reactors that were submitted to the laboratory for further cultural tests were found negative.

The hatchery reported evidence of CRD lesions in progeny from this flock when the birds were 9 months of age. A partial flock test at this time revealed 21 percent reactors.

The serum-plate test of the 476 birds selected for flock replacements at 10 months of age revealed 24 percent reactors. A total of 297 negative birds was retained from this group for pedigree hatching of flock replacements. As an additional precaution, these birds were placed on an 8-week antibiotic regimen of weekly intramuscular injections of 250 mg of duo-streptomycin sulfate (dihydrostreptomycin sulfate and streptomycin sulfate). Hatching eggs were saved one week following their first antibiotic injection.

At 12 months of age, the second test of the selected birds revealed 6 doubtful reactors. These birds were removed from the flock. On final test, at 13 months, only 3 doubtful reactors were detected among the 237 birds that remained. These birds were also removed from the flock.

Cull chicks and pipped eggs from these 297 birds selected from group A were examined for gross lesions of CRD. The examination of the first four hatches yielded the following results: 91 cull chicks, no lesions; 126 embryos from pipped eggs, 3 slight lesions, 2 marked lesions. The examination of three hatches, obtained following the removal of reactors detected by the second retest of these selected birds, gave the following results: 58 cull chicks, 1 marked lesion; 73 embryos from pipped eggs, 3 slight lesions, 3 marked lesions. The results of examination of cull chicks and pipped eggs from the last two hatches, obtained following the removal of the reactors detected by the final serological test of the selected birds, were as follows: 55 cull chicks, 2 slight lesions; 57 embryos from pipped eggs, no lesions.

A total of 3303 chickens, progeny of group A, was maintained on the farm as replacements for the following year. At 3 to 4 months of age, 1872 birds were tested by the rapid-serum-plate test. Three reactors were detected and one of these was positive on subsequent cultural tests. The total flock test, conducted when the birds were 5 to 7 months of age, revealed one doubtful reactor. PPLO was not isolated from this bird. No reactors were detected by a final test of 639 birds at 8 to 10 months of age that were selected to produce the flock replacements. The examination of cull chicks and pipped eggs obtained from these birds yielded negative results.

The group B birds remained serologically negative for CRD during the 12-month period of this study. Cull chicks and pipped eggs obtained from this group were negative for gross lesions of CRD.

Experiment 7. In the eight commercial flocks that were vaccinated for ND and IB, a random sample of birds was serologically tested for CRD at periodic intervals. On pre-

vaccination tests, the results were negative in all flocks. Four flocks revealed a few CRD reactors at the first postvaccination test. At the second postvaccination test, all eight flocks revealed evidence of CRD. In six flocks, the majority of the birds that were tested reacted. Similar results were obtained on subsequent tests in these six flocks. The majority of the birds tested in the remaining two flocks became positive within 5 and 7 months, respectively, postvaccination.

The susceptible controls that were placed in these vaccinated flocks 1½ to 4 months postvaccination were tested at frequent intervals for serologic evidence of CRD transmission. Evidence of CRD was detected in these controls within 1 to 2 months following placement in the flocks. The majority of the controls in seven flocks were positive within 3 to 4 months following introduction into the respective flocks. In the eighth flock, the majority of the control birds did not become positive until the 7th month after placement. The vaccinates in this flock also showed a slow serological response.

DISCUSSION

In experiment 1, the attempt to demonstrate incubator and brooder transmission of PPLO from infected to noninfected progeny failed. While only limited evidence of CRD infection was noted in embryos that failed to hatch and in one day-old chick from the positive flock, no further evidence of the disease was detected among the progeny that were reared from either the positive or negative flocks. Apparently the organism can be shed in the egg but its likelihood of initiating a generalized flock infection, even when the birds are stressed, may not occur in some instances, as was demonstrated by these negative results.

The results of egg transmission of PPLO in a flock in which the disease has subsided as compared with a flock manifesting an acute outbreak, appear most interesting. The transmission was very evident in the embryos from the flock that had experienced the more recent infection. In the chicks reared from this group, approximately 50 percent manifested signs of CRD and essentially 100 percent exhibited lesions,

in contrast to questionable evidence in the progeny from the flock in which the disease had stabilized. This latter group produced results somewhat similar to those obtained in the progeny from the positive flock cited in experiment 1.

In experiment 3, it was again demonstrated that in a flock in which the disease has subsided one finds little evidence of CRD in the embryos or cull chicks, while the chicks that are reared appear to be negative even when subjected to certain stressing agents.

The results of experiment 4 are of interest in that the elimination of the organism in the progeny hatched from the same flock of yearlings as reported in experiment 2 was considerably less than was observed 6 weeks previously. The chicks from this source failed to transmit the disease to chicks from a negative flock when hatched in the same incubator. It appears that partial recovery of the flock during this 6-week period had a definite lowering influence on the egg transmission rate of the organism.

In experiment 5, it was observed that transmission through cohabitation occurred without producing generalized clinical manifestations of the disease, and that essentially all the negative birds developed specific agglutinins within a 3-month period. Similar findings have also been observed in commercial flocks. The infection may spread through a flock manifesting little or no clinical evidence of the disease. On the other hand, in some flocks the disease may spread rapidly with pronounced clinical manifestations as was cited in the older birds of experiment 2.

Cultural results obtained with birds and fresh eggs revealed that the PPLO was not recovered from all specimens even though the birds proved to be consistently serologically positive. A much higher percentage of positive cultures was obtained from the birds than from the fresh eggs. The large number of positive cultures obtained from the negative contact birds, 7 days after the start of the experiment, was not expected. Some of these cultures were inoculated into embryonated eggs and typical lesions of CRD were observed. Twelve months later, approximately 50 percent of the cultures taken

from the remaining WL hens yielded PPLO. Relative to the fresh eggs cultured, only a small number were found positive and were laid by a few WL hens; whereas, none of those cultured from the BR hens yielded the organism. These results are difficult to evaluate considering the variation in the isolations obtained with the methods used. Similar observations have been made by Fabricant *et al.*[5]

Positive evidence of CRD in embryos and chicks was found in fewer hatches from the naturally infected group than from the contact group. This suggests that the birds which had experienced the more recent infection are likely to shed the organism more frequently in the egg. However, it is equally significant that the naturally infected group also was shedding the organism in the egg after having contracted the infection more than 12 months previously. These results would suggest that the chances of obtaining negative progeny from serologically positive flocks might be greater with flocks in which the disease has stabilized, than in flocks that have experienced a recent infection. Also, it appears that flocks with a long history of positive serologic findings should not be considered incapable of eliminating the organism through the egg or transmitting the infection to negative birds through cohabitation.

The naturally infected hens and the contact WL hens both manifested a marked resistance to a challenge inoculation with a pathogenic PPLO; whereas, the susceptible controls exhibited typical signs and lesions of the disease. The degree of resistance manifested by serologically positive birds is difficult to explain since some birds were still capable of shedding the organism. Also, flocks found to be serologically positive will remain so for many months. The question may be raised, what is responsible for the positive serology in these birds, since in most cases cultural and other tests fail to detect the organism.

The pattern of transmission of PPLO obtained in the commercial breeding flock in experiment 6 was somewhat different than observed in previous studies. The birds investigated were derived from breeding flocks that revealed

a low rate of egg transmission, based upon examination of cull chicks and embryos. It was anticipated that the progeny from this flock would not reveal CRD infection. The results of the serologic tests conducted, two prior to, and one after sexual maturity, suggested that the flock might be free of PPLO. However, subsequent observations revealed that the flock was infected but not to the degree that is frequently observed in field flocks. It was evident from these results that the infection was of such a nature in the early stages that it was not detectable by the test. It is also of interest that the random test of birds in all the pens revealed serologic evidence of infection.

It is significant that through continuous serologic testing and removal of reactors, a group of breeders was obtained that produced a CRD-negative flock. However, it should be recognized that this selected group of breeders produced PPLO infected eggs, as confirmed by gross CRD lesions in a few cull chicks and embryos. Although the final serologic test of the selected breeders was essentially negative, the hatches obtained after this test still revealed questionable evidence of PPLO infection in the cull chicks. The serologic testing of the immature progeny from these selected breeders revealed only slight evidence of PPLO infection and as the birds matured no conclusive evidence of CRD was noted either by serologic tests or by examination of cull chicks and embryos obtained from the eggs hatched from these birds. These results suggest that the establishment of a CRD-negative flock from breeders of questionable CRD status may be a tedious procedure since the infection may be dormant in the progeny and following successive negative serologic tests, the disease may later become apparent. However, other investigations have revealed that once a flock is free of the disease, it may remain so for several years and possibly longer.

The results obtained in experiment 7 reveal that PPLO infection was not detectable by serologic test in chicks approximately 4 weeks of age. However, after the vaccination for ND and IB, evidence of CRD infection did appear. In some flocks clinical signs of the disease were noticeable. The

results in these field flocks were different than those discussed in experiment 6. It appears that when progeny is derived from a breeding flock in which the majority of the birds are serologically positive that such progeny is apt to develop generalized CRD infection following stressing and usually before the birds attain sexual maturity. In such flocks when CRD-negative stock is introduced after sexual maturity, the infection may spread to these birds in one to two months. However, there may be exceptions as was pointed out in two flocks. This experiment confirms work reported previously by Van Roekel *et al.*[10]

The results reported in experiment 7 and others definitely indicate that PPLO is egg-transmitted, and that in the majority of flocks the infection does not spread through the flock until the birds are 4 weeks or older in age. It is recognized that there may be exceptions to this mode of spread. However, it should also be pointed out that once infection commences to spread within a flock, it may involve 100 percent of the birds in the flock. Serologic testing in such instances is not effective in checking or eliminating the disease.

SUMMARY

Negative results were obtained from the limited trials conducted on incubator and brooder transmission of CRD. Transmission by cohabitation was demonstrated in sexually mature chickens.

Egg transmission may be of a higher rate in flocks that have had a recent episode with CRD than in flocks in which the disease has become stabilized. Pathogenic avian Mycoplasma may be recovered more easily from recently infected flocks than from flocks manifesting few or no clinical signs and a prolonged positive serologic history. A serologically positive flock should be considered as a potential source of CRD for its progeny.

Infection may spread within a flock with slight or no clinical manifestations. In most flocks, clinical signs are usually evident and the entire flock becomes serologically positive. However, in some flocks a static level of infection

may occur as based on serological findings. In such cases, the removal of reactors may be effective in elimination of the infection from the flocks.

Serologically positive birds should not be introduced into negative flocks and conversely, serologically negative birds should not be introduced into positive flocks.

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HATCHERY SANITATION AS A CONTROL MEASURE FOR ASPERGILLOSIS IN FOWL*

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HATCHERY sanitation has hitherto been concerned with the control of enteric, bacterial and respiratory viral pathogens. Recently, however, high mold populations in commercial egg incubators were reported by Wright, Anderson and Epps.[7] The presence of molds was detected by routine, microbial analysis of fluff samples. Moreover, the presence of certain molds, particularly *Aspergillus spp.* often coincided with increased embryo mortality and clinical respiratory ailments, unthriftiness, and increased chick and poult mortality within the first 10 days of age. A similar condition was reported by Eggert and Barnhart[3] who noted a predisposition to *Aspergillus fumigatus* in young chicks, the progeny of a flock with aspergillosis, which was brooded on wire.

Concern over the increase in mycotic invasion of older fowl has been voiced by Couch[2] who claims that administration of antibiotics is a major influence on this phenomenon. However, this does not explain the reason for this type of disease in chicks and poults under 10 days of age nor does it relieve the hatchery manager of his sanitary obligations. The claim by Watkins[5] that 10 percent of all deaths in chicks under 14 days old are caused by aspergillosis indicates the economic importance of this disease. The spores of *Aspergillus spp.* are ubiquitous in their distribution and are present perenially but the presence of large numbers of these spores in fluff samples cannot be overlooked, especially since aspergil-

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losis is readily transmitted to young fowl by air pollution techniques according to O'Meara and Chute.[4]

The purpose of this investigation was to assess the significance of *Aspergillus fumigatus* in hatching compartments.

MATERIALS AND METHODS

Experiments were designed to study mold invasion of eggs during incubation, mold invasion of embryos, aspergillosis in chicks infected as embryos and aspergillosis in chicks exposed to spores during hatching.

The first experiment was designed to determine if mold mycelium could penetrate an egg during ordinary incubation. Fifteen dozen fresh chicken eggs were obtained from the Department of Poultry Science. These eggs were placed in setter trays so that alternate eggs were cracked at a point adjacent to a sound egg. Before placement in the trays, the cracked area of the appropriate eggs was daubed with a cotton swab which was rolled in petroleum jelly before dipping it into a supply of spores of *Aspergillus fumigatus*. Twelve additional eggs were placed in the trays so that they had no contact with cracked eggs.

Incubation was continuous and samples consisting of six cracked and six sound eggs were removed daily for microbial examination. They were surface-sterilized, broken out into jars and incubated for 3 days at 40 C. The cultures were examined macroscopically for mold growth. Culture work was done in a mold-free cabinet with all the material inside and fumigated with 2 ml of formalin and 1½ grams of potassium permanganate per cubic foot. The effectivity of all aseptic techniques was tested and proven adequate.

Experiment 2 was based on the results of the previous experiment. It was designed to allow time for penetration of developing embryos by mold mycelium before hatching commenced. Hence, 287 eggs were incubated in a mold free atmosphere for the first 10 days. One-half of these eggs was from unmated birds because they were to be cracked at the time of exposure to spores of *Aspergillus fumigatus*. Cracked eggs were not used if the membranes were ruptured during

the cracking process. Exposure was achieved by swabbing the cracked area with a suspension of spores and and egg albumen. During the incubation, eggs were removed for culture as in the first experiment. The remaining eggs were candled at transfer time in an effort to detect molds and to remove the cracked ones.

The fertile eggs were transferred to hatching trays and isolated by placing them into separate incubators which had been fumigated previously. The hatching was arranged so that six different groups of chicks were obtained. These groups are listed in table 1.

TABLE I

The hatching plan for experiment 2

Group #	Treatment
1	Fertile eggs in contact with cracked contaminated eggs from 10th day to 19th day of incubation, then removed and disinfected before hatching
2	The same treatment as group 1 except that the cracked eggs were not removed prior to hatching
3	Embryos as in group 1 but in addition a cracked moldy egg was also placed in this hatching compartment
4*	Unexposed embryos until the 19th day, when a cracked, moldy egg was placed in the hatching compartment with them
5*	Normal unexposed chicks placed in the battery brooder along with a cracked moldy egg
6*	Healthy, unexposed control group

* The eggs for groups 4, 5 and 6 were incubated in machines in a separate building

The chicks which were produced in the six groups were placed in electrically heated battery brooders with ¼ inch wire mesh floors. Each battery brooder was placed into a cage constructed of wooden framework covered by clear plastic sheeting. One end was removable and contained two ventilation openings 8 x 20 inches which contained cotton filters (figure 1). Feed and water could be added from the outside so that the isolation of each group could be maintained throughout the experimental period. The cages were not

opened to remove the dead birds. At the end of 9 days' brooding, the birds were all killed and weighed. Samples of the feces from all cages were collected and examined for Aspergillus.

RESULTS

In the first experiment, mold penetration of eggs occurred within 10 days in an egg incubator after the eggs had been contaminated with spores of *Aspergillus fumigatus* mixed in petroleum jelly. Penetration of cracked eggs was demonstrable in this experiment one day sooner than in sound eggs. The entry of molds into the interior of eggs was enhanced

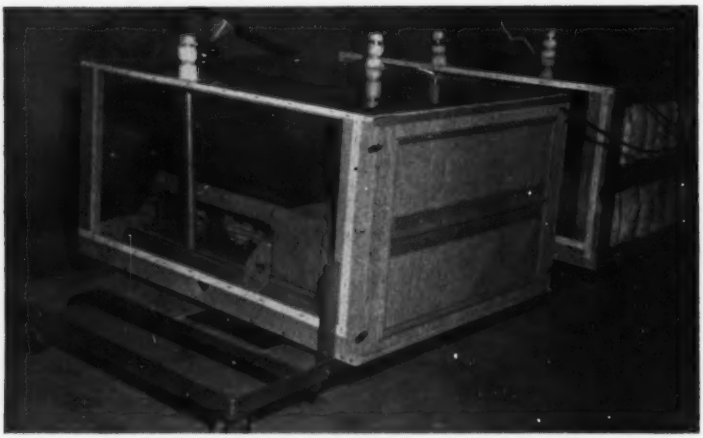

Fig. 1. Isolation units constructed of wood and plastic sheeting.

by cracking the shells before they were inoculated as shown by the presence of 76 and 20 mold infected eggs in the cracked and sound groups respectively. After 21 days' exposure of eggs to spores, extreme moldy conditions resulted whereby conidia could be poured from the air cell when opened (figure 2). The results of this experiment are presented in table 2.

In the second experiment, the first mold infected egg was detected in cracked and sound eggs on the 4th day and 5th day respectively, following surface contamination of the cracked area. This was 6 days less than the time required for

TABLE II

Daily cultural examinations of cracked and sound eggs after contamination with spore-petroleum jelly mixture

# Days incubation	#Eggs infected over # eggs examined	
	cracked	sound
10*	3/6	0/6
11	6/6	2/6
12	4/6	1/6
13	6/6	2/6
14	6/6	0/6
15	5/6	0/6
16	6/6	0/6
17	6/6	1/6
18	6/6	0/6
19	5/6	0/6
20	6/6	0/6 **
21	17/19	14/17(8/12)
Totals	76/85	20/83(8/12)

* no infected eggs prior to 10 days' incubation
** these eggs were not in contact with cracked eggs

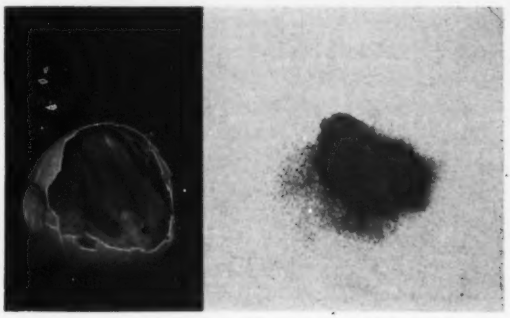

Fig. 2. Conida poured from a cracked moldy egg 21 days after surface contamination.

penetration by the mold in the first experiment. The shorter time required for penetration may be related to the materials used to bind the spores to the egg, petroleum jelly and egg albumen. In this experiment, cracks in the shells of eggs also enhanced the penetration by molds but sound shells, on the other hand, did not exclude them completely. These results of daily cultural examinations of six eggs from each group following contamination are presented in table 3.

TABLE III

Isolation of *A. fumigatus* from egg contents removed daily from an egg incubator

Days incubated	Days after contamination	Eggs infected/Eggs examined	
		cracked	sound
11	1	0/6	0/6
12	2	0/6	0/6
13	3	0/6	0/6
14	4	1/6	0/6
15	5	2/6	2/6
16	6	2/6	1/6
17	7	0/6	0/6
18	8	5/6	3/6
19	9	70/101	0/90*

* 90 eggs transferred to hatching compartments

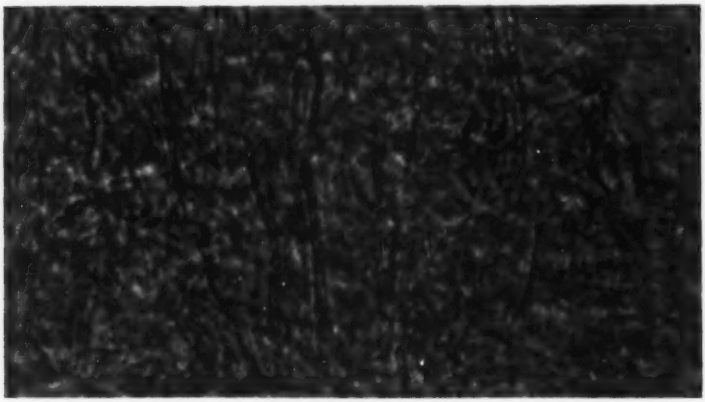

Fig. 3. Microphotograph of exudate from interclavicular air sac × 100.

On the 19th day of incubation, 90 eggs were transferred to hatching compartments. Sixty-seven chicks hatched according to the plan presented in table 1. One of the chicks was late hatching and was not included in the brooding experiment but was killed and examined. *A. fumigatus* was isolated from the lungs of this chick and what appeared to be mycelium could be seen in smears of exudate from the interclavicular air sac. A microphotograph of this material is presented in figure 3.

Chicks which had been hatched in a mold-free incubator were marked and mixed with those that had been exposed to molds in order to ascertain whether transmission would occur during hatching and brooding. Isolation cages were arranged to contain chicks according to the plan in table 4. There was a considerable variation in the number of chicks which hatched from the exposed embryos. This accounts for the difference in the number of birds placed in each cage.

TABLE 4

Arrangement of chicks in isolation cages

Cage #	# Chicks per cage*	Method of exposure to *A. fumigatus*
1	13/18	Embryonating eggs having 9 days' contact with cracked, contaminated eggs then hatched in a mold free atmosphere
2	9/9	Same as cage 1 except the cracked contaminated eggs were also placed in the hatcher
3	8/15	Same as cage 1 except an opened moldy egg was also placed in the hatcher
4	7/24	Embryos were unexposed until 19th day when they were placed in a hatcher with moldy egg similar to cage 3
5	22/–	Normal unexposed chicks placed in the isolation cage with a moldy egg similar to cage 3
6	22/–	Healthy unexposed controls

* Normal chicks/exposed during hatching

The cages were sealed after the chicks were placed in them. They were not opened during the 9-day brooding period, hence dead chicks could not be removed for cultural determinations. Clinical symptoms such as laboured breathing, flicking the head and gasping preceded death in most cases. Anal pasting was first noted at 5 days of age which is in accord with results reported by Eggert and Barnhart.[3]

At the end of the 9-day brooding period, all survivors were killed by asphyxiation with carbon dioxide, weighed and cultured. These data along with the daily mortality figures are presented in tables 5 and 6.

TABLE V

Mortality and isolation data for chicks exposed to *A. fumigatus* as embryos and chicks

Cage #	1	2	3	4	5	6
Temperature F.	98	98	93	93	93	93
Age in days	Mortality					
1	2	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	2	0	0
4	0	1	1	4	0	0
5	0	1	2	5	2	0
6	0	0	3	0	1	1
7	1	0	3	0	0	0
8	0	1	1	3	1	0
9	0	0	0	3	0	1
Totals*	1/2	0/3	2/8	0/17	4/–	2/–
Isolations*	1/1	2/1	1/3	4/4	5/–	0
Chicks per cage*	13/18	9/9	8/15	7/24	22/–	22/–

* number of normal chicks over the number exposed ones when brooding commenced.

TABLE VI

Weight and morbidity of chicks nine days of age

Cage #	1	2	3	4	5 ***	6
# Survivors*	12/16	9/6	6/7	7/7	18/–	20/–
Av. wt. of normals	103.3	79.8	92.7	96.6	80.3	95.6
Av. wt. of exposed	86.7	73.8	60.0	66.6	—	—
Total morbidity**	16.1	33.3	65.2	80.6	40.9	9.0

* normal/exposed, see table 1
** no. dead plus no. with mold infected lungs in per cent.
*** exposed to spores in brooder

In the analysis of the data presented in table 6, total morbidity represents the number of chicks which died during brooding plus the number of chicks from which *A. fumigatus* was isolated at the end of the experiment. It was stated earlier that the cages were not opened during the brooding period in an effort to prevent cross-infection between groups, hence, it was impossible to remove the dead ones. The highest mortality was experienced in cages 3 and 4 which included chicks hatched from embryos incubated both with and without

contact with cracked contaminated eggs. Both groups of chicks in these two cages were hatched in a compartment containing a moldy egg which had been broken open. While it is quite likely that some of the infection in group 3 may have occurred in the embryonic stage, it is quite evident that infection occurred during hatching as evidenced in the mortality in the individuals brooded in cage 4. The first exposure of this group to the mold was an opened moldy egg placed with them on the tray of the hatcher. Admittedly confinement was more precise in the hatching tray than in the brooder and therefore could have caused greater exposure of individuals to spores. On the other hand, there may be a considerable increase in resistance to infection among chicks during the first few hours after hatching. This is indicated by the fact that four of seven normal contacts with infected chicks yielded *A. fumigatus* upon culture of the lungs while the mold was only recovered from five out of 22 normal chicks brooded with a moldy egg in cage 5. The fact that a milder infection may have occurred in cage 5 resulting in spontaneous remissions should not be overlooked. If this does occur, it is further evidence that resistance to infection may increase in the first few hours after chicks are hatched.

It is impossible to calculate the effect of the higher temperature on the morbidity in cages 1 and 2. However it does establish the fact that embryos infected with *A. fumigatus* do hatch. The extent to which such embryos become infected with molds would be a function of the physical factors affecting sanitation surrounding the egg during incubation.

DISCUSSION

Since it has been shown that embryos become infected during incubation and since chicks are very susceptible to invasion by *A. fumigatus* during the hatching period, it is very important that this mold be prevented from achieving high spore "populations" in incubators. Fumigation of hatchers as outlined by Wright and Epps[6] is an aid although not a complete control for this situation. Everything possible should be done to prevent mold growth and thereby

reduce the number of spores released in the entire hatchery building. Moreover, it is also necessary to prevent spores of molds from accumulating in any part of the hatchery and equipment. This is necessary because the spores of *A. fumigatus* have remained viable at room temperature for at least 18 months in hatchery dust.

Furthermore, the invasion of the respiratory tract may not be the most serious aspect in the transmission of aspergillosis by hatcheries. Imprints of live chicks as they are removed from infected hatching compartments revealed that the exterior of the chicks was grossly contaminated by spores. This could lead to contamination of equipment and brooder pens. The contamination of litter by this means may be more serious than realized. Heretofore, litter has been labelled as the source of infection in most cases of aspergillosis as shown by the comprehensive review of the literature by Chute and O'Meara.[1] Results of these experiments indicate the importance of hatchery sanitation in the prevention of the contamination of chicks which may subsequently infect the litter.

As a result of the knowledge gained through these and other experiments, a number of recommendations are made in an effort to control transmission of aspergillosis and possibly other similar mycotic infections by hatcheries. These recommendations are by no means exhaustive but are intended rather to guide the formulation of practical plans.

1. Only clean eggs should be set and the setters kept clean at all times.
2. Avoid setting cracked eggs and unnecessary cracking should be prevented during incubation.
3. Cracked eggs should be removed at transfer time and if any moldy ones are seen the adjacent eggs should also be removed.
4. Moldy eggs should not be broken open in the hatchery.
5. The general atmosphere of the hatchery should be kept scrupulously clean at all times.
6. Adequate microbiological techniques should be used to determine the sanitary status of a hatchery rather than visual methods.

7. Sanitary requirements for hatcheries should be more realistic and be aimed at the control of mold as well as bacterial and viral populations.

ACKNOWLEDGMENT

The authors wish to acknowledge the assistance of J. B. Fischer, Mycologist, Ontario Department of Health, for the identification of the molds.

CONCLUSION

Aspergillosis in fowl is becoming an important factor in the economy of poultry production in this country as a result of recognized and obscure losses. Under experimental conditions, penetration of sound and cracked eggs by *Aspergillus fumigatus* occurred in the incubator. Transmission of the disease occurred during hatching. To minimize losses from the disease, more effective control over the sanitation of commerical hatcheries is recommended.

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EASTERN VIRAL ENCEPHALOMYELITIS IN CHUKAR (*ALECTORIS GRAECA*)

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EASTERN viral encephalomyelitis, formerly eastern equine encephalomyelitis, has been reported many times in horses, man, and various species of birds. Most species of birds have been infected experimentally and outbreaks have been reported in many types of wild birds. The principal vector is the mosquito. Many natural outbreaks of the arthropod-borne disease have been reported in pheasants, however, no mention is made of the chukar. *Culiseta melanura* and other species of mosquito known to be vectors have been found in our general area.

Beaudette and Black,[1] Beaudette and Hudson,[2] Van Roekel and Clark,[7] Simpson, Lewis, and Jaquette[6] reported natural outbreaks in pheasants reared on game farms. Kissling, *et al.*[5] reported the isolation of eastern viral encephalomyelitis virus from the blood of the purple grackle. Fothergill and Dingle,[4] as well as others, reported that a fatal disease of pigeons proved to be eastern viral encephalomyelitis.

This virus disease was diagnosed in horses on the Del-Mar-Va peninsula during the late summer and early fall of 1959. Twenty or more cases were reported from farms near the marshy areas of Wicomico, Worcester, and Somerset counties.

During the middle of September four 8-week-old chukar chicks were presented to the laboratory with a history of a disease of sudden onset and with sudden deaths. These chicks

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were hatched from breeders reared on the same farm the previous year. These adults had no history of disease that could be observed by the caretaker.

The birds were reared in pens with wire floors. All pens were raised about two feet above the ground. There were four pens of chukar chicks within a few yards of each other. Each pen consisted of an entire hatch. There were four groups, each hatched at a different time. The pens were within a few hundred yards of a fresh water stream that had numerous stagnant pools along its course that could make excellent breeding areas for arthropods, the vectors of eastern viral encephalomyelitis.

Only one pen of 100 chicks was involved in this outbreak. The other three pens failed to develop symptoms or mortality at that time or since. There were 700 quail reared on wire platforms in the same manner as the chukar. The quail pens were dispersed among the chukar pens, some adjacent to the pen of the sick chukars. No evidence of eastern viral encephalomyelitis was detected in these birds.

The first change observed by the caretaker was a bleaching out of the beaks and legs. The legs and beaks lost their shiny, glistening appearance. Next the birds appeared dull and listless. There was inappetance in the affected pen. Soon the birds fell over backward, much like "crazy chick disease" in chicks and later became completely prostrate. There was a marked contrast between the affected and unaffected pens.

Two birds died on the day symptoms were first observed. Mortality increased rapidly until a peak of 20 per day was reached on the 4th day after the onset and ceased completely 3 days following the peak. A 40 percent mortality occurred during the 7 days in which losses occurred.

Standard procedure used for poultry necropsy was carried out when these birds were presented to the laboratory. Bacteriological and parasitological examinations were negative. Since there were nervous symptoms in the live birds, brain tissue was collected for viral examination.

The tissue collected was ground and treated with 10,000

units of penicillin and 25 mg of streptomycin per ml and inoculated into the allantoic cavity of five 9-day-old chick embryos. The only embryo death occurred at the end of 48 hours post inoculation time. Material from this embryo was harvested and injected into a second series of eggs with no embryo mortality. No gross pathology was observed when the remaining embryos were examined.

Another sample of the brain emulsion was injected intracerebrally into young white mice all of which died within 48 hours. Brains from the mice were harvested and filtered with a Swinney type filter. This filtrate was injected subcutaneously into five one-day-old chicks. These chicks developed nervous symptoms and died within 48 hours. This is quite characteristic of eastern viral encephalomyelitis. Part of the mouse brain filtrate was injected into young mice subcutaneously. These mice developed nervous symptoms with 100 percent mortality within 56 hours.

Samples of the original brain material, as well as parts of the other materials, were frozen and submitted to the Grayson Laboratory, where Dr. Frank Hetrick, Department of Veterinary Science and Microbiology, University of Maryland, identified the virus as that of eastern viral encephalomyelitis. This identification was made by the standard methods of serological typing.

The flock was visited seven weeks after the disease had occurred to see what effect it had had on the birds. The birds in the affected pen had been through a severe body molt that had started while the disease was in progress. Growth had been very severely affected since birds in the affected pen were much smaller than those in the unaffected groups.

At the same time, sera was collected from six birds from the affected pen. These birds had hemagglutination-inhibition titres ranging from 1/40 through 1/1280+. These titres are quite in agreement with titres produced in birds that have been artificially infected for about the same period of time. A hemagglutination-inhibition titre of 1/10 is an indication of exposure to eastern viral encephalomyelitis.[3]

SUMMARY

The virus of eastern viral encephalomyelitis was isolated from a group of chukar partridge reared on a game farm.

Only birds in one of four pens developed symptoms of the disease.

There was a 40 percent mortality in the affected pen.

Growth was inhibited in the affected group.

Hemagglutination-inhibition titres seven weeks after the outbreak indicated exposure to eastern viral encephalomyelitis.

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SOME OBSERVATIONS OF THE PROPAGATION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS IN TISSUE CULTURE†

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THE virus of infectious laryngotracheitis (LT) produced cytopathogenic effect (CPE) and plaques in chick-embryo fibroblast and respiratory epithelium.[1] However, Buthala, *et al.*[3] was unable to find CPE with LT virus in chick-embryo kidney cells, although the virus proliferated in the cell culture.

The present study had three objectives: 1. to study the effect of LT virus in chick-embryo kidney cells and its ability to produce CPE and plaques, 2. to attempt the isolation of the virus by plaque method from chickens artificially or naturally infected with LT virus, 3. to study the differences between plaques formed by LT virus, chick-embryo-lethal-orphan (CELO) virus and Newcastle disease (ND) virus.

MATERIALS AND METHODS

Virus strains. Three strains of LT virus, one strain of ND virus and one strain of CELO virus were used. All were obtained from the source indicated, and were received in allantoamnionic fluid. All were propagated in 10 or 11-day-old chick embryos.

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Infectious laryngotracheitis virus. All LT virus strains were chick embryo adapted live vaccines. They were passed twice in chick embryos before use in tissue culture. They were Vineland, DPL and ASL, obtained from Vineland Laboratory, Vineland, New Jersey; Delaware Poultry Laboratory, Millsboro, Delaware and American Scientific Laboratory, Madison, Wisconsin, respectively.

Newcastle disease virus. The strain of ND virus used was GB Texas, 1948.[2]

Chicken-embryo-lethal-orphan virus. The Phelp strain of CELO virus[11] used in this study was obtained from Dr. Vance J. Yates, Chairman, Department of Animal Pathology, University of Rhode Island, (URI) Kingston, Rhode Island.

Preparation of cell culture. The procedure used in the preparation of cell cultures was a modification of the method of Younger[12] for growing monolayer monkey kidney tissue. Kidneys were removed aseptically from 20 day old chick embryos, washed in phosphate-buffered saline[10] (PBS) and trypsinized with warmed (37 C) 0.25 percent trypsin for one hour. The cell suspension was centrifuged for 10 minutes at 1000 rpm. The packed cells were resuspended in growth medium which consisted of Hank's basic salt solution (HBSS) enriched with 0.5 percent lactalbumin hydrolysate, five percent calf serum, 0.5 percent of 7.5 percent sodium bicarbonate and one percent penicillin-streptomycin solution (P-S). This P-S solution contained 10,000 units of penicillin and 10,000 mcg of streptomycin per ml. After a second round of centrifugation and decantation, the cells were resuspended in growth medium at the rate of 1.0 ml of packed cell to 100 ml of medium. This cell suspension was filtered through two layers of sterile gauze. A cell count was made with a hemacytometer and growth medium was added to make the final cell concentration of 500,000 to 700,000 per ml.

The final suspension was distributed in 2.0 ml amounts in Leighton tubes and in 9.0 ml amounts in three ounce prescription bottles. Tubes and bottles were sealed with white rubber stoppers. At the end of the second or third day,

the growth medium was removed and replaced with fresh medium. A continuous sheet of cells appeared on the third or fourth day and it was then ready for seeding of the virus.

Immune and normal sera. Normal serum was obtained from six-week-old chickens which had been kept in isolation since hatching and which had shown no respiratory symptoms during that period. Immune serum against infectious bronchitis (IB) virus was obtained from twelve-week-old chickens. These birds were inoculated intratracheally at 8 weeks of age with 2×10^3 embryo lethal doses of Van 8 (repository #41) strain of IB virus. This IB strain was described by Chang.[4] The birds showed respiratory symptoms two days after inoculation and were kept in isolation until bled.

The LT anti-serum was prepared in rabbits using the Vineland virus. The rabbits were inoculated intravenously with 0.25 ml of the virus in allantoamnionic fluid every 3rd day and were bled 2 weeks after the fourth inoculation.

Immune serum for ND virus came from the URI Animal Pathology Department's stock preparation, obtained from chickens that survived an artificial challenge of GB Texas ND virus. Immune serum for CELO virus was obtained from Dr. Vance J. Yates. The serum was prepared in rabbits.

Plaque method — Agar overlay technique. The agar overlay technique was that described by Hsiung and Melnick.[8] When the cells in the bottles had grown into a complete monolayer, the nutrient fluid was removed. Virus samples in 0.2 ml amounts of serial tenfold dilutions were introduced into each bottle. Three or five bottles were inoculated for each dilution. The inoculated bottles were then incubated at 37 C for one hour after which 9.0 ml of overlay agar medium was added to cover the cell sheet. This medium contained HBSS with 1.5 percent agar, 5 percent calf serum, 0.5 percent lactalbumin hydrolysate, 0.0025 percent neutral red, 0.07 percent $NaHCO_3$ and 1 percent P-S. After the agar had solidified the bottles were turned over and incubated at 37 C. Observation of the bottles for plaques was made daily over a fluorescent illuminator.

Experimental procedures. Cytopathogenicity. All strains of LT virus were tested by inoculating 1.0 ml of allantoamnionic fluid into each bottle of embryo kidney cell culture and 0.1 ml into each Leighton tube. Tubes and bottles were examined daily.

Primary isolation with plaque method. Three samples of tracheal exudates in nutrient broth were obtained from the diagnostic laboratory, Department of Animal Pathology, University of Rhode Island. Each sample was collected from birds with a natural outbreak of LT disease. The diagnosis of LT disease was based upon history, post mortem lesions and lesions produced in embryonated eggs. Six susceptible Rhode Island Red chickens, 16 weeks of age, were used for each strain of LT virus. The six birds for one strain were divided into two groups. Group 1 was inoculated intratracheally with 0.3 ml of virus infected allantoamnionic fluid. Group 2 was given no virus and was placed in direct contact with the inoculated birds. Seventy-two hours after inoculation, all birds were killed, and their tracheas were removed and opened aseptically. The tracheal exudate of each bird was harvested with a sterile cotton swab and placed in a tube containing 3.0 ml of nutrient broth. Tracheal exudates of each group were pooled and stored in a deep freeze at -10 C until used.

At the time of inoculation, the tracheal exudates were thawed, and 0.5 ml of each sample was added to 1.0 ml of P-S and 3.5 ml of PBS. This constituted the 10^{-1} dilution. Further tenfold dilutions were made with PBS. These were inoculated in bottles and overlaid as already described.

Virus neutralization test. One half of a ml of undiluted heat inactivated serum, which was either made up of normal, anti-LT, anti-ND, anti-IB or anti-CELO immune serum was mixed with 0.5 ml of P-S and 1.0 ml of LT virus (DPL strain). The LT virus contained at least 2×10^2 plaque forming units. The mixture was incubated at 37 C for 30 minutes and 0.2 ml of the mixture was inoculated into each bottle to be overlaid.

RESULTS

Cytopathogenic effect and plaque production of LT virus on embryo kidney cells. Of the three strains of LT virus, all produced CPE on the first passage in embryo kidney cells. The CPE developed within 24 to 48 hours after inoculation. In 48 hours after inoculation, most of the sheet of cells had shrunk and disappeared. Round cells which had lost their cytoplasm were located in clusters around the periphery of the sheet of cells. There were many dead cells floating in the medium.

Plaques were also produced by all three strains of LT virus. The rate of growth and morphology of plaques were indistinguishable among the three strains. Approximately 72 hours after inoculation, indistinct, pin point areas appeared on the sheet of stained cells. After 96 hours, these spots enlarged and appeared as circumscribed round dots. Their diameter measured less than one mm. At this time, the plaques could be recognized and counted. The titer of plaque forming units (PFU) of LT virus from undiluted allantoamnionic fluid, usually reached 10^4. The plaques increased in size, reaching a diameter of 2-3 mm on the 8th day and 3-4 mm on the 12th day. The edge of the plaques also became irregular resulting in indistinct boundaries.

Primary isolation. Primary isolation from birds inoculated with three strains of LT virus was successful. Plaques appeared 96 hours after seeding with the ASL and DPL viruses and 120 hours after seeding with Vineland virus. The titers of PFU of the viruses from the tracheal exudated were approximately 10^5 with ASL, 10^4 with DPL and 10^3 with Vineland. From the contact birds only Vineland Strain produced plaques with a titer 10^1.

Similarly, plaques were produced from all three tracheal exudates from birds naturally infected with LT disease. Plaques appeared on the 4th or 5th day after inoculation. There were no differences between the plaque morphology of the three isolates.

Virus neutralization test. The ability of the LT virus to

produce plaques was neutralized by anti-LT serum. This neutralization was not seen when normal, anti-ND, anti-CELO or anti-IB serums were used.

Differences were found between the plaques produced by LT, CELO and ND virus as shown in table I.

TABLE I

Differences in plaques formed by infectious laryngotracheitis virus, CELO virus and Newcastle disease virus

Virus	Days plaques first appear after inoculation	Size of 12th day after inoculation	Boundary
Infectious laryngotracheitis virus	3 - 4	3 - 4 mm	Irregular indistinct
CELO virus	5 - 6	1 - 2 mm	Sharp distinct
Newcastle disease virus	2	6 - 7 mm	Distinct

DISCUSSION

The plaque formation of LT virus provides a practical method in the identification and titration of the virus. Since differences in rate of growth, morphology and size of plaque, formed by LT, ND and CELO viruses, do exist, a differential test by this method is practical. The LT virus was isolated directly from artificially or naturally infected birds by the plaque method, demonstrating the possibility of using this method in primary isolation.

Strains within a single type of virus may differ in their plaque producing capacity. This has been shown by Granoff[5], Hsiung *et al.*[9] and Henderson *et al.*[7] Further study of plaque formation by various strains of LT viruses is indicated.

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SUMMARY

1. Cytopathogenic effect and plaques in chick-embryo kidney cells were produced by three strains of infectious laryngotracheitis (LT) virus.

2. The ability of the LT virus to produce plaques was neutralized by specific anti-LT serum.

3. Plaque method could be used in primary isolation from birds artificially or naturally infected wtih LT virus.

4. Differences in rate of growth, morphology and size were found in plaques formed by LT, chick-embryo-lethal-orphan (CELO) and Newcastle disease viruses.

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A BACTERIAL AGENT CAUSING BLUECOMB DISEASE IN TURKEYS

1. ISOLATION AND PRELIMINARY LABORATORY INVESTIGATIONS

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BLUECOMB disease of turkeys is a condition affecting turkeys of all ages which has as its chief characteristic a catarrhal enteritis and which presents no pathognomonic features. Usually a diagnosis is based on history, post-mortem observations and the absence of known bacterial pathogens or protozoa. The clinical features and gross pathology of the disease have been described by Pomeroy and Sieburth.[2] Sieburth and Johnson[3] have proposed that this condition be called transmissible enteritis of turkeys. A filterable agent has been reported as the cause of the disease by Tumlin *et al.*[4] and by Sieburth and Johnson.[3]

A disease which in all essential respects conforms with the clinical and pathological picture described by the above workers, and which we regard as bluecomb disease, was a relatively minor problem in adult flocks of Ontario turkeys until recently. However, during the brooding season of 1959 the disease was diagnosed in young poults. Subsequently, several hatcheries were affected and the problem became the most serious one affecting young poults in this province. The present study was designed to characterize the agent responsible for

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the disease as it occurs in Ontario and the following report will describe the isolation and some of the cultural characteristics of a small, Gram-negative, anaerobic bacterium which, when administered to susceptible poults, reproduces the disease.

Initially, several groups of turkey poults originating from one hatchery were submitted to the diagnostic laboratory for examination. There was a history of inappetence, decreased water intake and a tendency to huddle close to the heat. Post-mortem examination revealed the intestines to be distended with watery, gaseous material. The ceca were enlarged and contained light brown, foamy fluid. In many cases, the gizzards contained only litter. Microscopic examination of sections of intestine stained with hematoxylin and eosin revealed a catarrhal enteritis. Mortality ranged from 10 to 15 percent depending on the age of the birds.

Routine bacteriological culture techniques failed to reveal bacteria of probable significance. Intestinal scrapings were negative for protozoa. Transmission of the condition to healthy poults was readily accomplished by the oral administration of a crude suspension prepared from the intestines of diseased birds. A clinical diagnosis of transmissible enteritis was made, and the following studies were commenced.

EXPERIMENTAL PROCEDURES

The poults used for transmission experiments throughout this study were from a hatchery known to be clinically free of enteritis. Serial blind passages of intestinal suspensions prepared from control groups of these birds and administered orally to successive groups provided evidence that birds originating in this hatchery were not latent carriers of the condition.

Transmission in poults. In order to establish that the condition was experimentally transmissible, intestine taken from field cases was treated as follows: a 20 percent suspension of intestines and their contents was prepared in tryptose phosphate broth by milling in a Waring Blendor for 2 minutes. The suspension was centrifuged for one half hour at 3800

rpm and the supernatant was used as inoculum. The same procedure was followed in all subsequent preparations of intestinal suspensions for bird to bird transmission experiments.

Each of ten, one-day-old poults received 1.0 ml of the supernatant administered into the crop with an 18 gauge teat cannula. Four poults were sacrificed at 7 days of age and the remainder at 14 days of age. No significant gross signs of the disease were found, but an intestinal suspension was prepared from the four poults which had been sacrificed at 7 days of age and this was used to inoculate twenty, day-old poults. There were single deaths in this group on the 4th, 5th, 7th, 8th and 10th days after inoculation. On post-mortem examination, portions of the intestines of these poults were seen to be distended and contained watery, gaseous material. The remaining 15 birds were sacrificed at 2 weeks and six of them showed similar signs.

Similarly, ten, one-day-old poults were inoculated as above for a third serial passage. One poult died in 7 days and one died 9 days after inoculation. The remainder were sacrificed at 19 days and those intestines which showed lesions were used to prepare an inoculum for the fourth passage in which 24, day-old poults were inoculated. The first death occurred at 3 days and all but one were dead by the 9th day. Lesions were the same as those described above.

Subsequent passages resulted in mortality which began on the second day following inoculation and usually reached 100 percent by the 10th day. Intestines showing typical lesions and frozen at -20 C have been found capable of producing clinical signs, mortality and lesions when administered to poults after a storage period of 7 months.

Propagation in chick embryos. Intestines taken from the 10th serial passage in poults were used in an attempt to propagate the causative agent in embryonated eggs. Small portions of affected intestines from several birds, totalling approximately 8 cm in length, were cut into small fragments and gently shaken in 20 ml of tryptose phosphate broth. In order to avoid the use of antibiotics, attempts were made to render the suspensions bacteria-free by filtration through

Millipore filters. Difficulty was experienced passing the crude suspension through the fine filters. A procedure found satisfactory was that of serially passing the filtrate through membranes of graded porosity starting with Millipore membrane filters of pore size 1.2 μ and proceeding through 800 mμ, 650 mμ and 450 mμ. The final filtrate was used to inoculate six-day embryonated eggs by the yolk sac route. Each embryo received 0.2 ml. The embryo mortality of this and four successive passages is shown in table I. The agent has been passed a further 19 times by the yolk sac route with essentially the same mortality pattern.

Titrations in chick embryos by the yolk sac route showed that infective yolk had an embryo LD_{50} of $10^{-4.5}$ and that the embryo LD_{50} of allantoic fluid and embryo liver was less than 10^{-1}.

TABLE I

Embryo mortality

Passage	No. of embryos inoculated (via yolk sac)	Inoculum	Embryo deaths Days post inoculation 1	2	3	4	5	6	7
1	10	Intestinal filtrate	1		1	4	3		1
2	10	Yolk from passage #1			1	5	4		
3	9	Yolk from passage #2	1	5	2	1			
4	6	Yolk from passage #3			2	3	1		
5	8	Yolk from passage #4		1	2	3	2		

Filterability. Several experiments using Millipore membrane filters were carried out. Infective yolk was diluted with an equal quantity of tryptose phosphate broth and successively passed through filters of pore size 1.2 μ, 800 mμ, 650 mμ, 450 mμ and 300 mμ. The integrity of the 450 mμ and 300 mμ filters was checked by attempting to pass *Serratia marcescens* through them and subculturing the filtrate in fresh broth. The unfiltered mixture and the filtrates from the 450 mμ and the 300 mμ filtration were used for inoculation of chick embryos by the yolk sac route. In one experiment a Seitz EK filtrate was also tested.

In most filtration experiments, embryo mortality, fol-

lowing inoculation with yolk filtered through the 450 mμ filter, was delayed 2 to 4 days in comparison with mortality in embryos given unfiltered yolk, but ultimately, mortality in the groups reached about the same level. There were no specific deaths in chick embryos receiving filtrates passed through the 300 mμ filter. There was no embryo mortality in the one instance where the Seitz EK filtrate was used as an inoculum.

Antibiotic sensitivity. Chemotherapeutic agents were tested at various levels in chick embryos for activity against the agent. The following final concentrations were used:

Penicillin 1000 units per ml plus streptomycin 1 mg per ml.

Penicillin 1,000 units per ml.

Streptomycin 1 mg per ml.

Achromycin 50 μg, 100 μg, 1 mg and 5 mg per ml.

Chloromycetin 5 mg per ml.

Erythromycin 5 and 25 mg per ml.

Spontin 1, 5 and 25 mg per ml.

Neomycin 500 μg, 1 mg and 5 mg per ml.

Dilutions of the drugs were prepared in tryptose phosphate broth and each was mixed with an equal amount of infective yolk. The mixture was incubated at room temperature for 15 minutes and 0.2 ml of the mixture was inoculated into the yolk sacs of six-day embryonated eggs. In addition, control groups of embryos were inoculated with equivalent levels of the antibiotic alone.

It will be seen in table 2 that typical embryo mortality patterns resulted except in the case of neomycin where embryo mortality was reduced. Control embryos receiving the drugs alone are not shown. Deaths were negligible and were considered non-specific.

Pathogenicity of chick embryo-propagated agent for poults. Yolk from the fifth embryo passage was given orally to a group of ten, day-old poults. When sacrificed 6 days post inoculation, they showed distended intestines with watery, gaseous contents. An intestinal suspension prepared from these was used to inoculate a second series of day-old poults

TABLE II

Effect of various antibiotics on the lethality of the agent in chick embryos

Drug	Final concentration per ml	No. of embryos inoculated (via yolk sac)	Embryo mortality: Days post inoculation 1	2	3	4	5	6	7	8	9	10	11	Survivors
Infective yolk only		10		2	3	2			2	1				0
Penicillin and streptomycin	1000 units 1 mg	6			1				2		1	2		0
Streptomycin	1 mg	7				1			3	1	2			0
Penicillin	1000 units	8		1	1	2			2	1				0
Achromycin	50 μg	10			6	2	2							0
Achromycin	100 μg			1	2	2	1	3	1					0
Achromycin	1 mg	10	1	3		1			3	1	1			0
Achromycin	5 mg	10	1	1					1	3	2	1	1	0
Chloromycetin	5 mg	6				3			2		1			0
Erythromycin	5 mg	10		4	4		1		1					0
Erythromycin	25 mg	9							2	3	1	1	1	1
Spontin	1 mg			2	1	4	2	1						0
Spontin	5 mg	10	1	1	1	1	4	1	1					0
Spontin	25 mg	10			5	2		2	1					0
Neomycin	500 μg	8	1						2	2	2	1		0
Neomycin	1 mg	8				1		1				2	1	3
Neomycin	5 mg	8			1									7

and lesions were evident within 4 days. An intestinal suspension from the second group was used to inoculate a third group and lesions were evident within 6 days. There was no mortality in the first passage but evidence of the disease was seen in the birds sacrificed. Five of 30 poults died in the second passage and the remaining birds showed characteristic signs. A third poult to poult passage produced evidence of infection but no mortality. A suspension of intestines prepared from the third passage poults and passed through a 450 mμ pore size Millipore filter, when injected via the yolk sac, produced a mortality patttern in chick embryos similar to that seen in table 1.

In a further trial designed to determine if the yolk sac-

propagated agent still retained pathogenicity for poults, a 16th embryo passage was chosen. Yolk was administered to 30, day-old poults. Three poults died. Of ten birds sacrificed on the 6th day, the intestines of six showed characteristic signs and the intestines of four were normal. The remainder, sacrificed on the 14th day were normal. Affected intestines from birds sacrificed on the 6th day was serially passed a further five times through groups of poults. Each passage produced light mortality and a variable morbidity involving about half the birds examined. There was no apparent increase in ability to produce morbidity or mortality. The agent was recovered in chick embryos from an intestinal filtrate prepared as above from intestines of the fourth poult to poult passage. The pattern of embryo mortality was as previously seen.

Isolation of a bacterium. Yolk from infected embryos had been tested from time to time for the presence of bacteria on the following media; PPLO agar, tryptose yolk agar, and sheep blood agar with and without a *Staphylococcus aureus* streak. The cultures had been incubated aerobically, anaerobically, and with 10 percent carbon dioxide. These methods failed to reveal the presence of bacteria. For reasons that will be discussed, attempts to culture the infective agent on bacteriological media were continued.

In one of these later trials infective yolk was plated on tryptose chicken blood agar and the plates incubated aerobically, anaerobically and in an atmosphere of 10 percent carbon dioxide. The anaerobic plates when examined in 96 hours, revealed small, dew-drop like colonies. A Gram-stained smear showed the colonies to be composed of tiny, Gram-negative, poorly staining, somewhat pleomorphic rods. The appearance of colonies was a fortuitous incident which could not be repeated at will, and growth in fluid media containing 0.1 percent agar was investigated. When thioglycollate broth or cystine trypticase agar was inoculated with yolk or intestinal filtrate, growth was evident after 72 to 96 hours incubation at 37 C., as a hazy outline outside the line of inoculation. Inoculation was accomplished using a Pasteur

pipette to deliver approximately 0.1 ml of inoculum into the medium near the bottom of the tubes. Growth was less evident after the first transfer to fresh media, and barely perceptible after the second transfer. There was no growth after further transfers employing these media. Incorporation of 10 percent horse serum had no apparent effect in stimulating growth.

The glucose-cysteine semi-solid medium of Lewis *et al.*[1] when modified by the incorporation of 10 percent horse serum or 10 percent fresh yeast extract and 10 percent blood extract was found to support not only initial growth but to be satisfactory for maintaining growth of the organism. Up to 7 serial passages have been performed employing this medium, growth being evidenced by a very faint clouding which becomes apparent after 3 to 5 days of incubation at 37 C.

Pathogenicity of the bacterium for poults. The bacterium, isolated in glucose-cysteine medium from a filtrate prepared by passing a suspension of affected intestines through the 450 mμ pore size Millipore filter, was transferred twice more through fresh glucose-cysteine medium at 3-day intervals. The dilution of original, intestinal material accomplished in the three broth transfers was approximately 10^{-8}. After 72 hours incubation, 1 ml of broth per poult was administered to ten, day-old poults. There was no mortality, nor were gross signs of infection found when the poults were sacrificed 10 days post inoculation. Ten, day-old poults were maintained separately as controls.

Suspensions of intestines from both inoculated and control groups were prepared separately and used to inoculate a group of nine and a group of five, day-old poults respectively. The infected group of nine poults was sacrificed at 8 days post inoculation. The intestines of three appeared normal, three showed marked lesions and three showed lesions highly suggestive of the disease. The five poults receiving the control intestine suspension were sacrificed at 9 days post inoculation and their intestines were normal. A third passage was carried out in the same manner employing one group of twenty and one group of five, day-old poults. All but four poults in the infected group showed evidence of the disease. The bacterium

was recovered in media from the intestines of this group using the filtration technique previously described. The intestines of those poults receiving the intestinal suspension from control poults remained normal and attempted isolation of the organism was unsuccessful.

DISCUSSION

The disease in young poults whether contracted experimentally or naturally presents the same picture. Inappetence, huddling, and decreased water intake are among the signs first noted. Droppings are usually quite loose, frequently light brown and may appear somewhat foamy when fresh. On post-mortem examination, a catarrhal enteritis is seen and portions of the intestine are distended with watery, gaseous material. The ceca are frequently enlarged and contain light brown foamy fluid. The other internal organs appear normal.

Since the disease which we have studied in Ontario is clinically, and in its gross pathology, identical with that described by Pomeroy and Sieburth[2] as affecting poults in Minnesota we consider them as probably the same entity. This is supported by experiments paralleling theirs in which a filter passing agent was found capable of transmitting the condition. The facility of handling Millipore filters prompted their use in preference to Selas, Berkefeld or Chamberland filters. As in the work reported by Tumlin *et al.*[4] and Sieburth and Johnson,[3] the infectious filtrate was bacteria-free when checked by routine procedures for aerobes and anaerobes. Since, in our studies, the integrity of the filters in all crucial experiments was tested with *Serratia marcescens*, we were led to believe initially that we were dealing with other than a bacterial agent and chick embryo infectivity trials were undertaken.

An intestinal suspension passed through a 450 mμ pore size Millipore filter produced mortality in chick embryos when inoculated into the yolk sac and the infection was found to be transmissible serially through chick embryos by this same route. The allantoic route proved unsatisfactory. At this point we were still unable to demonstrate bacteria in

the yolk although it was infectious both for poults and chick embryos.

As has been reported above, the infective yolk had an embryo LD_{50} of $10^{-4.5}$ and the embryo LD_{50} of allantoic fluid and embryo liver was less than 10^{-1}. This concentration of infectious property in the yolk sac and its size as determined by filtration tended to suggest a psittacoid or a rickettsia-like agent. However, Giemsa and Macchiavello stains of yolk sac impression smears failed to reveal intracellular organisms. Tests with various antibiotics had shown that the agent was resistant to all of them at the levels employed with the exception of neomycin. The consideration of these facts led to a more intensive investigation of the possibility that a bacterium was the etiological agent.

Although it was found that colonies appeared occasionally when infectious yolk was plated on tryptose chicken blood agar plates, colonization could not be repeated at will and the use of thioglycollate broth and cystine trypticase agar was investigated. These were found to be quite satisfactory for isolation but a modified glucose-cysteine broth was satisfactory both for isolation and maintenance. The bacterium has been isolated from several intestinal filtrates of experimentally infected birds and from two field cases presented for diagnosis. Isolation attempts from experimental, control poults have been negative.

Transmission of the disease to poults was accomplished with yolk from a sixteenth serial passage in chick embryos and also with a broth culture of the bacterium derived from an intestinal suspension of affected birds. The possibility exists that a virus was carried concomitantly with the organism through the embryo passages, but the broth culture used was a serial passage representing ultimately a 10^{-8} dilution of intestinal contents. This renders it less likely that a virus agent was carried. We believe that the evidence presented indicates that a bacterium is the etiological agent of bluecomb.

SUMMARY AND CONCLUSIONS

A Gram-negative anaerobic bacterium isolated by means

of filtration from poults affected with bluecomb has been grown in the yolk sac and produces mortality in embryonated eggs.

Infectious yolk has been shown to produce an enteritis in turkey poults clinically indistinguishible from transmissible enteritis or bluecomb.

Similarly, the bacterium, grown in glucose-cysteine broth, was capable of inducing the disease in poults and the organism was recoverable from their intestines. It could not be recovered from control poults in which serial passage of control intestines was carried out.

Filtration trials indicated that the bacterium will pass through a Millipore filter of 450 mμ pore size but not through a filter of 300 mμ pore size.

The bacterium is resistant to penicillin, streptomycin, tetracycline, erythromycin, chloromycetin and spontin but is somewhat sensitive to high concentrations of neomycin.

It is the authors' belief that a small, Gram-negative, anaerobic, pleomorphic rod is the etiological agent of transmissible enteritis or bluecomb of turkey poults.

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PLASMA ENZYME LEVELS IN CHICKENS, A MEASURE OF DRUG INDUCED PATHOLOGY*

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SERUM enzyme level studies are a part of established clinical procedure in the diagnosis of many pathological conditions. According to several authors[6,15,32] the two principal mechanisms by which abnormally high amounts of enzyme are released into the circulation are: (1) cellular degeneration and lysis, which is due either to a pathological condition or an increased metabolic rate or, (2) secretion of the enzymes by the intact cells either as a physiological process or as a result of a pathological condition.

At present there are many drugs on the market, which are used in many conditions of poultry. These drugs are injected into, or fed to the birds for therapeutic reasons, and also as growth or production stimulants. Methods to evaluate the effects of these drugs on the birds, are mostly confined to growth and production records. In cases of suspected, grave, toxic effects, pathological studies are made. Our study was an attempt to see whether slight physiological and pathological changes from normal may be detected in a group of experi-

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mental birds, using enzyme level studies as a criterion of these changes.

LITERATURE REVIEW

The three enzymes, serum glutamic-oxaloacetic transaminase (GOT), lactic dehydrogenase (LHD) and alkaline phosphatase (Alk. Phos.) have been studied extensively. The increase of these enzymes in the circulation in the course of many pathological conditions has been reported by various investigators. Normal levels of the various enzymes in both serum[37] and tissues[6,18] of a number of experimental, domestic animals[8,9] and man[37] have been established. Hess[15] has carried out a study of normal and pathological levels of LDH in human serum. It is his contention that normal enzyme concentrations in the serum are maintained mainly by their release from disintegrating erythrocytes. He further states that due to normally low turnover of other tissue cells (muscle, nerve tissue), little enzyme from these tissues is normally present in the serum.

Pathological levels of the three enzymes under study are reported by numerous investigators. High plasma LDH values are associated with pathological conditions such as hemolytic anemia and aleukemic type of leukemia[4]; increases are also noted in cases of myocardial infarction and various neoplastic conditions. Increases of serum GOT in cases of tissue damage and necrosis have been reported, as an example; in local tissue injury, myocardial injury[3,21] white muscle disease of lambs and calves[5,20,32] liver pathology [7,10,11,17,22,23,26] muscular dystrophy of poultry[9] and experimentally produced pathology using toxic chemicals. Increases of serum Alk. Phos. levels are seen in osteogenic sarcoma,[29] osteitis fibrosa cystica, rickets, osteomalacia and conditions such as myeloblastosis, jaundice and various liver disorders. Motzok from the results of experiments with normal and rachitic chicks[26,27,28] concluded that increases of plasma phosphatases in rickets were due to the leakage of the enzyme from the bones to the tissues.

Increased levels of serum enzymes have been reported in experimentally induced conditions. Toxic chemicals[23] have

been used to cause increase of serum GOT. Drabkin[12] found increases of Acid and Alk. Phos. in alloxan- induced diabetes in rats. Alk. Phos. has been found to be decreased in the serum during fasting and increased when fat was given in the diet of rats and man.[33]

MATERIALS AND METHODS

Seventy birds were used in the three experiments. Forty of these birds were White Leghorns and the rest Nichols 88's and Vantress X White Rock. These birds were of mixed sexes and seven weeks old at the beginning of the experiments.

The drugs used in this study were: oxytetracycline*, terephthalic acid*, NF260 brand of nitrofurazone**, sulfabromomethazine***, zinc bacitracin****, chlortetracycline, hygromycin B***** and hydroxyzine hydrochloride*.

One form of injectable oxytetracycline and one form of water soluble N260 were used. The rest of the drugs were mixed with a basal ration and fed continuously. The basal ration was a mixture of a growing mix and starter mash as manufactured and sold by Wirthmore Feeds Inc., Waltham, Mass.

Blood samples were obtained by cardiac puncture. The blood was heparinized with heparin diluted 1:20 with water (1,000 units per ml.=10 mgms.) Microhematocrits and hemoglobin levels were determined. Next the plasma was centrifuged off and the enzyme activity levels were determined.

Plasma enzyme methods were based on the work of McDaniel[24] on normal plasma enzyme levels of chickens. The GOT assay method was adapted from the method of Karman *et al.*,[18,19] as modified by Steinberg *et al.*[31] The reagents were obtained from the Warner-Chilcott Co. LDH values were obtained by using the method of Wrobleski and LaDue.[37] Plasma Alk. Phos. reaction rates were determined as described in the Sigma Technical Bulletin #104.[30] In the case of Alk. Phos.,

* Supplied by the Charles Pfizer Co.
** Supplied by the Eaton Drug Co.
*** Supplied by Merck & Co. Inc.
**** Supplied by Commercial Solvents Corp.
***** Supplied by Eli Lilly Co.

the plasma was diluted 1:10 with water after the work by McDaniel.[24]

At the completion of each experiment the birds were necropsied. Portions of liver, kidney, lung, pancreas, duodenum, spleen and heart were fixed in 10 percent formalin, sectioned and stained with hematoxylin and eosin.

The three experiments were arranged as follows:

Experiment I. Forty Leghorns were used, divided into four lots and treated as follows:

Lot 1—Four subcutaneous injections of oxytetracycline were made at weekly intervals with the following dosages used on each bird: 100 mg, 100 mg, 200 mg, and 250 mg.

Lot 2—Controls on basal ration.

Lot 3—NF260 at 0.15 percent concentration was mixed with the basal ration.

Lot 4—NF260 was added to the drinking water at a rate of 3 grams per gallon.

This treatment was continued for approximately one month. The birds were bled four times at fairly regular intervals, with the first bleeding one week after the start of the experiment. Four GOT, three LDH, one Alk. Phos., three hemoglobin and four hematocrit determinations were made.

Experiment II. Thirty Vantress X White Rock and Nichols 88 birds were divided into lots of five and treated as follows:

Lot 1—Basal ration alone.

Lot 2—Basal ration + oxytetracycline at 600 grams per ton.

Lot 3—Basal ration + 0.5 percent sulfabromomethazine.

Lot 4—Basal ration + terephthalic acid, 20 pounds per ton.

Lot 5—Basal ration + bacitracin, 300 grams per ton.

Lot 6—Basal ration + 0.15 percent NF260.

This treatment was continued for approximately 3 weeks. The birds were bled at three fairly evenly spaced intervals, starting one week after the start of the experiment. Three GOT, three LDH, two Alk. Phos., one hemoglobin and three hematocrit determinations were made.

Experiment III. Thirty Vantress X White Rock and Nichols 88 birds were divided into six lots of five and treated as follows:

Lot 1—Basal ration + 0.5 percent sulfabromomethazine.

Lot 2—Basal ration + chlortetracycline, 600 grams per ton.

Lot 3—Basal ration + hydroxyzine hydrochloride, 15 grams per ton.

Lot 4—Basal ration + terephthalic acid 20 lbs. per ton.

Lot 5—Basal ration + hygromycin B, 4 lbs. per ton.

Lot 6—Basal ration alone.

These birds were on this treatment for a period of 2 weeks. They were bled twice. One GOT, two LDH and one Alk. Phos. determinations were done.

RESULTS

The results of the plasma enzyme determinations summarized in tables 1, 2, and 3, and show that significant enzyme level differences from control groups were obtained through the use of high levels of some of the drugs.

The values for each group were analyzed. Statistical

TABLE I

Experiment 1—Means of plasma enzyme values***

Treatments	GOT	LDH	Alk. phos.
Control	157.4±14.9	825.8±183.0	29.6±12.4
Oxytetracycline injectable	146.4±11.0	906.0±166.0	26.2±20.3
NF260 in feed	** 209.4±18.0	** 1038.5±104.0	36.9±27.1
NF260 in water	** 186.4±24.0	* 1032.3±156.0	* 53.7±33.0

* = Significant at 5% probability level.
** = Significant at 1% probability level.
GOT = Activity in units per ml. of plasma.
LDH = Activity in units per ml. of plasma.
Alk. Phos. = Expressed as Bessey-Lowry units (Sigma).
*** = Values are in the averages of mean readings for all bleedings from each bird in each group. The standard deviation on this average value is indicated.

TABLE II

Experiment 2—Means of plasma enzyme values***

Treatments	GOT	LDH	Alk. phos.
Control	145.1±19.1	596.1±114.0	43.3±23.5
Oxytetracycline in feed	155.8±11.5	** 696.6±124.0	23.0±10.0
NF260 in feed	** 187.6±15.6	** 958.2±181.0	48.4±20.5
Sulfabromomethazine in feed	149.7±20.7	** 825.5±47.0	36.3± 9.7
TPA in feed	173.5±35.5	* 820.1±172.0	47.1±14.0
Zinc Bacitracin	141.1±18.5	** 926.0±155.0	51.6±17.0

* = Significant at 5% probability level.
** = Significant at 1% probability level.
GOT = Activity in units per ml. of plasma.
LDH = Activity in units per ml. of plasma.
Alk. Phos. = Expressed as Bessey-Lowry units (Sigma).
*** = Values are in the averages of mean readings for all bleedings from each bird in each group. The standard deviation on this average value is indicated.

TABLE III

Experiment 3—Means of plasma enzyme values***

Treatments	GOT	LDH	Alk. phos.
Control	191.0± 7.8	1078.9±120.0	25.3± 7.7
Sulfabromomethazine in feed	211.5±44.0	1203.2±228.0	18.7± 8.5
Chlortetracycline in feed	206.2±42.0	* 1370.7±101.0	25.6± 9.2
Hydroxyzine Hydrochloride in feed	* 248.9±48.0	1164.9± 89.0	21.9± 9.3
TPA in feed	185.3±30.6	1541.6±517.0	30.3± 9.6
Hygromix in feed	187.3±36.0	** 1329.1±119.0	29.5± 8.1

* = Significant at 5% probability level.
** = Significant at 1% probability level.
GOT = Activity in units per ml. of plasma.
LDH = Activity in units per ml. of plasma.
Alk. Phos. = Expressed as Bessey-Lowry units (Sigma).
*** = Values are in the averages of mean readings for all bleedings from each bird in each group. The standard deviation on this average value is indicated.

analysis* was done on the mean values of each bird. Significant increases in plasma GOT were obtained in chickens fed NF260 and hydroxyzine hydrochloride. The increases in plasma LDH in chickens fed NF260, oxytetracycline, sulfabromomethazine, terephthalic acid (TPA), zinc bacitracin, chlortetracycline and hygromycin B (hygromix) were also significant. Plasma Alk. Phos. increased significantly in chickens fed NF260. Thus in chickens fed NF260 there was a significant increase in the activity of all three enzymes.

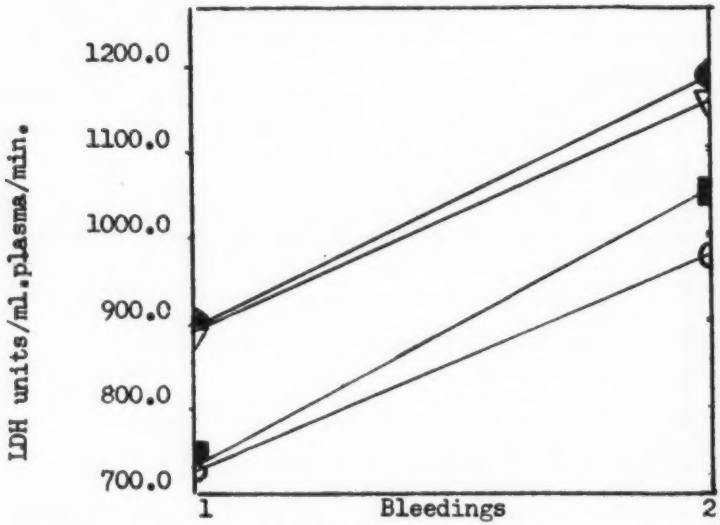

Fig. 1. Experiment #I, LDH Plasma Values*
Control = O NF260 in feed = ●
Oxytetracycline inj. = ▪ NF260 in water = Δ
*Values are a mean of enzyme activity levels of each group at one bleeding.

Definite pathological changes were seen in the birds receiving the different drugs. The greatest pathological changes were seen in chickens fed sulfabromomethazine, hygromix and more particularly NF260 in which group there was a significant increase in all three enzymes. The pathology

* Student's unpaired "t" test was used as described by Snedecor G. W., Statistical Methods, Iowa State College Press, Ames, Iowa (1956).

was most evident in liver and kidney. Cloudy swelling of parenchymal cells, fatty degeneartion, necrosis, heterophilic and mononuclear infiltrations in the portal areas, were seen in the liver. Kidney changes were; cloudy degenerations of tubular cells, cell atrophy, cystic degeneration, necrosis and the presence of calcified casts in the tubules; Grossly, a loss of weight and extreme dehydration was evident in birds on high levels of NF260. A loss of weight was apparent in the birds fed high levels of sulfabromomethiazine. No significant changes from control values were apparent in the hematocrit and hemoglobin values.

DISCUSSION

The choice of the drugs used in this study was arrived at by considering a number of drugs commonly used by poultry growers. It was hoped that by using these drugs at three times the maximum recommended level and continuously, a varied response would be arrived at, both, in the plasma enzyme concentrations and pathology caused. The results tend to show that the plasma enzyme changes are a fairly early indication of changes occurring in various organs. The pathological changes in the liver and kidney caused by the high levels of NF260 and sulfabromomethazine were accompanied by high levels of plasma enzyme activities.

In the statistical evaluation of enzyme activities, means of individual birds were used. This was considered to be the most reliable method of estimating the difference of response in the course of the experiments. Figures 1 and 2 show that even though a correlation could be seen in some cases as in LDH values in Exp. I, at other times no simple relationship could be seen as exemplified by the unpredictable fluctuation of the LDH values in figure 2. We were interested in total enzyme activity present during the course of our experiment. We believe that the fluctuations were caused by the indivdual response of the birds to the drugs and varied with the stress and the susceptibility of the experimental animal to it.

The scope of this study was too limited to draw definite conclusions about the effects of any of the drugs under the

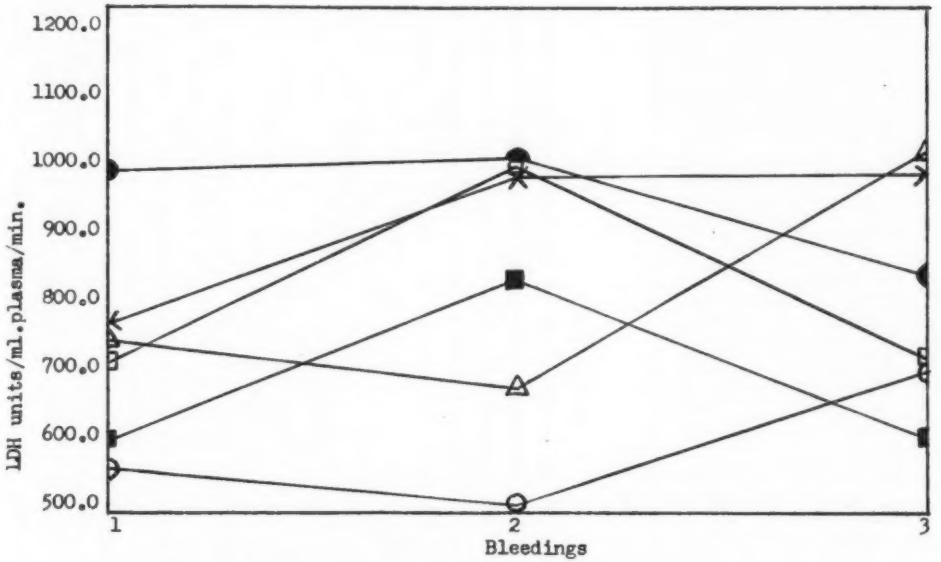

Fig. 2. Experiment #II, LDH Plasma Values*
Control = O TPA in feed = □
Oxytetracycline in feed = ▪ Zinc Bacitracin in feed = ×
Sulfabromomethazine in feed = Δ NF260 in feed = ●
* Values are a mean of enzyme activity levels of each group at one bleeding.

experimental conditions described. However, the correlation between plasma enzyme increases, pathology and loss of condition by the birds was quite evident. Study of plasma enzymes in toxicity work seems to be an approach that would merit a wider study.

SUMMARY

The levels of three enzymes, glutamic-oxaloacetic transaminase, lactic dehydrogenase and alkaline phosphates have been followed in the plasma of experimental chickens subcutaneously injected with or fed different drugs in their ration or drinking water at high levels for a period of 2 to 4 weeks. These conclusions were arrived at on the basis of the results obtained:

1. Increases in enzyme levels in this experiment were correlated with the toxic reactions induced in some vital organs, notably liver and kidney.
2. In our experiments, increases in lactic dehydrogenase levels were the earliest indication of tissue damage due to toxicity.

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CONTROL OF CHRONIC RESPIRATORY DISEASE II. THE EFFECT OF LOW CALCIUM DIET, TEREPHTHALIC ACID AND CHLORTETRACYCLINE*

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TWO of the major accomplishments of the research on infectious synovitis were the studies on comparative ac-

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tivity of the tetracycline antibiotics[10-13] and the stimulation of research on ways to potentiate antibiotic activity.[1,5,6,8,9,12,14] To date, the dietary efficiency of these broad spectrum antibiotics has been increased in three ways: (a) use of terephthalic acid (TPA), (b) use of rations low in divalent ions and (c) by supplying supplemental calcium as calcium sulfate instead of calcium carbonate.[2,8,9,14]

From the results of Olson and Shelton,[3] the minimum concentration (125 gm/ton of feed) of chlortetracycline (CTC) used was as effective in preventing the retardation of growth in *Mycoplasma* (MP) inoculated birds as higher concentrations. In order to prevent the development of chronic respiratory dsease (CRD), high concentrations of CTC and potentiation are necessary.[7] It was desirable, therefore, to determine the minimum concentration of CTC which would prevent the retardation of growth due to CRD and to determine the effect that relatively high and low concentrations of calcium have on the efficacy of CTC.

MATERIALS AND METHODS

For this trial, 150 White Rock X Vantress chicks were placed into each of 32 pens with 150 square feet of floor space. Half of the chicks were obtained from flocks known to have a high transmission rate of CRD and the other half from flocks known to have a low transmission rate of CRD. The chicks were evenly mixed in each pen and the treatments were randomly distributed throughout the house. The chicks in sixteen of the above pens were fed a relatively high calcium diet (1.4 percent) and those in the other sixteen pens were fed a relatively low calcium diet (0.85 percent). At each calcium level, replicate treatments were maintained at concentrations of 0, 25, 50 and 100 grams CTC per ton of feed and then repeated with 0.5 percent TPA.

At ten days of age, 50 birds in each pen were inoculated intrasinusly with a 72 hour broth culture of MP (WVU 1791) containing approximately 2×10^9 organisms per milliliter. The chicks were vaccinated with bronchitis and Newcastle disease dust vaccine at eleven days of age.

The chicks were observed for signs of CRD and all birds that died were autopsied. Air sac lesions were determined in autopsied birds and in slaughtered birds on the dressing line. The birds were weighed at 9 weeks of age and feed conversions calculated. At ten weeks of age 20 birds from each replicate were tested for CRD using the serum plate agglutination test and ten birds were cultured for MP as described previously.[3]The colonies on PPLO agar were examined for pathogenic strain morphology.

RESULTS AND DISCUSSION

Definite clinical signs of CRD were present in some birds of all pens and evidenced by rales which continued beyond the usual time of a vaccination reaction.

TABLE I

Average weights and feed conversions of duplicate treatments as influenced by CTC, TPA and calcium content of ration*

Level of CTC gms/ton	Calcium content, percent			
	0.85	1.4	0.85	1.4
	Average weight, lbs.		Feed conversion	
0.5% TPA				
0	2.78	2.88	3.17	2.91
25	3.07	3.07	2.93	2.97
50	3.20	3.12	2.81	2.96
100	3.24	3.16	2.77	2.85
No TPA				
0	2.94	2.91	2.95	2.78
25	3.06	2.98	2.93	2.93
50	3.07	3.10	2.83	2.88
100	3.26	3.13	2.75	2.94

* Nine weeks reults.

The average weights and feed conversions are shown in table 1. According to the analysis of variance, the mean average weights supported at all levels of antibiotics were significantly ($P < .50$) greater than those when no CTC was fed. Also the weights of the birds on the 100 gram level

were significantly higher than those on the 25 gram level. No significant differences were observed for differences in calcium content, TPA content or interactions of these.

Feeding CTC in the low calcium ration reduced feed conversion values. One hundred grams of CTC were needed for a significant effect. On the high calcium ration, the levels of CTC used did not improve feed conversion over that of the treatment without CTC. No effect due to TPA were detectable.

TABLE II

Mortality, air sac lesions and reactors as influenced by calcium Content, TPA and CTC in the ration

Level of CTC gms/ton	Calcium content, percent					
	0.85	1.4	0.85	1.4	0.85	1.4
	Mortality, %		Air sac lesions, %		Reactors*, %	
0.5% TPA						
0	8.4	6.8	13.3	9.0	100	98
25	4.4	6.3	5.3	5.0	90	98
50	2.4	2.4	1.3	3.7	65	80
100	6.7	3.3	2.0	1.3	18	50
No TPA						
0	4.7	6.6	3.0	2.0	100	100
25	3.7	4.0	6.3	4.3	100	100
50	5.1	4.0	0.7	7.0	95	100
100	3.2	6.3	6.3	6.7	85	85

* Based on a serum plate test of 13 percent of the birds.

The percentage of mortality, air sac lesions and reactors are shown in table 2. The variation which occurred within the various treatments was too great for significant effects to be detected relative to mortality and air sac lesions. The results of the serum plate agglutination test showed that CTC levels had little effect toward reducing the percentage of reactors. With 0.5 percent TPA in the ration, significant reductions in the percentage of reactors occurred. At the 0, 25, 50 and 100 grams of CTC plus the TPA, the percentage

of reactors were 100, 90, 65 and 18, respectively, when the 0.85 percent calcium ration was fed. With the 1.4 percent calcium ration, 98, 98, 80 and 50 percent of the birds showed positive agglutinations at the above concentrations of CTC, respectively.

Detailed results of the tracheal cultures are not shown. It was interesting, however, that the highest number of isolations of MP occurred from those groups which received the 100 gram concentration of CTC. As the amount of CTC in the ration decreased, the number of isolations decreased. This is consistent with the results of Olson *et al.*[4] who showed that a great reduction in the percentage of tracheal isolations of MP occurred in experimental birds between the 6th and 10th weeks. Those groups on the lower levels of CTC probably developed a greater degree of resistance; therefore, the elimination of MP was greater. Two reasons can be advanced to account for the effect of CTC on MP isolation and antibody response; (1) the CTC controlled the MP to a limited extent and there was less antigenic stimulation to antibody producing sites and (2) the CTC interfered with antibody production directly.

From the results presented, the value of low concentrations of CTC in the ration is not very great. From 50 to 100 grams are needed to protect the birds from the growth retarding effects of MP. That CTC is more effective at low calcium levels was aptly demonstrated by the percentage of reactors which occurred at the two calcium levels where TPA and different levels of CTC were fed.

ACKNOWLEDGMENTS

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SUMMARY

From fifty to a hundred grams of chlortetracycline per ton of feed were needed to protect chicks from the growth retarding effects of Mycoplasma. Other than this effect on

growth rate, the response to such low concentrations was of questionable value. Chlortetracycline, plus 0.5 percent terephthalic acid, reduced the percentage of Mycoplasma reactors more effectively in the ration containing 0.85 percent calcium than in the one containing 1.4 percent calcium.

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CONTROL OF CHRONIC RESPIRATORY DISEASE III. ISOLATION OF MYCOPLASMA AS INFLUENCED BY POTENTIATED CHLORTETRACYCLINE AND TIME INTERVAL AFTER EXPOSURE

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CHEMOTHERAPEUTIC agents have been widely used in an attempt to control chronic respiratory disease (CRD). The efficacy of these drugs has been expressed primarily on the basis of increased feed efficiency and weight gain and by decreased mortality, air sac lesions and respiratory signs. Generally, the effect of these drugs on Mycoplasma (MP) has not been considered.

From the reviews by Hofstad[1] and Olesiuk *et al.*[2,3] of the literature on the value of chemotherapeutic agents for the control of CRD, antibiotics have been of questionable value. The potentiation of tetracycline antibiotics by feeding terephthalic acid (TPA), low calcium rations, and the use of calcium sulphate in place of calcium carbonate has increased interest in the use of antibiotics for the control of CRD.[7] Olson *et al.*[4] and Peterson and Hendrix[6] in experimentally exposed chicks found that 250 grams chlortetracycline (CTC) per

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ton of feed plus 0.5 percent TPA prevented the development of agglutinins for MP and markedly reduced isolation of MP from the trachea. Mycoplasma were not isolated from birds given 375 grams of CTC plus 0.5 percent TPA,[4] but under different environmental conditions, MP were isolated from birds that had been fed 500 grams of CTC and 0.5 percent TPA.[5]

The expriment reported in this paper was designed to obtain: (1) more basic information on the course of CRD in a broiler flock as determined by isolation of MP from the trachea at two week intervals and (2) the effect of 400 grams of CTC per ton of feed with and without 0.5 percent TPA given continuously and for different time intervals on (a) isolation of MP, (b) weight gain and (c) feed efficiency.

MATERIALS AND METHODS

Into each of 32 pens (150 sq. ft.) were placed 100 White Rock-Vantress cross chicks. The treatments were randomly distributed throughout the house. The basal ration as fed was calculated to have 0.8 percent calcium and 0.8 percent phosphorus. At 2 weeks of age, each chick was inoculated intrasinusly with 0.25 ml. of a 72 hour broth culture of MP (WVU 1791) that contained about 11×10^7 organisms per ml. The chicks were dust vaccinated for Newcastle disease at 2 weeks and for bronchitis at 4 weeks of age. The design of the experiment and medication periods are shown in table 1.

Tracheal swabs were taken from 100 birds selected at random before inoculation and cultured for MP using the technique previously described,[4] except the third passage was made in maltose PPLO broth and thioglycollate media. If no growth occurred in the thioglycollate medium and the PPLO maltose broth became acid, this was considered a positive isolation. Three to four cultures from each treatment were plated to check for characteristic pathogenic strain morphology. Tracheal cultures from 20 percent of the chicks were made from treatments 1, 2, 3, and 4 at four weeks, from treatments 1 through 6 at 6 weeks, and from all treatments on the 8th and 10th weeks. Birds were weighed and feed conversions were calculated.

TABLE I

The experimental design showing the periods during which medication (M) was administered*

Treatment*	Weeks medication given (M)									
	1	2	3	4	5	6	7	8	9	10
1	M	M	M	M	M	M	M	M	M	M
2	0	0	0	0	0	0	0	0	0	0
3	0	0	M	M	M	M	M	M	M	M
4	0	0	M	M	0	0	0	0	0	0
5	0	0	0	0	M	M	M	M	M	M
6	0	0	0	0	M	M	0	0	0	0
7	0	0	0	0	0	0	M	M	M	M
8	0	0	0	0	0	0	M	M	0	0

* Two replications for each treatment were maintained and the birds in each except control groups recieved either 400 grams CTC per ton of feed (16 pens) or the same level of CTC plus 0.5% TPA (16 pens).

RESULTS AND DISCUSSION

The course of CRD in this flock and the influence of the various treatments is illustrated by the isolation percentage shown graphically in figures 1, 2, 3, and 4. In figures 3 and 4 the treatments were started two weeks before the first cultures were taken.

No isolations of MP were obtained from 100 tracheal swabs taken at the time of inoculation at two weeks of age. Two weeks after inoculation, 100 percent of the tracheal swabs from the nonmedicated groups revealed MP. The following isolation percentage of MP for the other periods were as follows: six weeks—95, eight weeks—40 and ten weeks—15.

When CTC was fed continuously (figure 1) it made little difference whether CTC was started at day old or at time of inoculation, if TPA was given. Without TPA the continuous medication from one day of age was consistently better in reducing the number of isolations than when CTC was started at time of inoculation. Feeding CTC during the third and fourth weeks (figure 2) reduced the percentage of isolations from 100 to 60 and with TPA to 40 at the end of four weeks; however, removal of the CTC allowed the infection to continue as if no antibiotic had been given during this two-week period. The CTC apparently interfered with the immune response and the additional stress of bronchitis vaccination

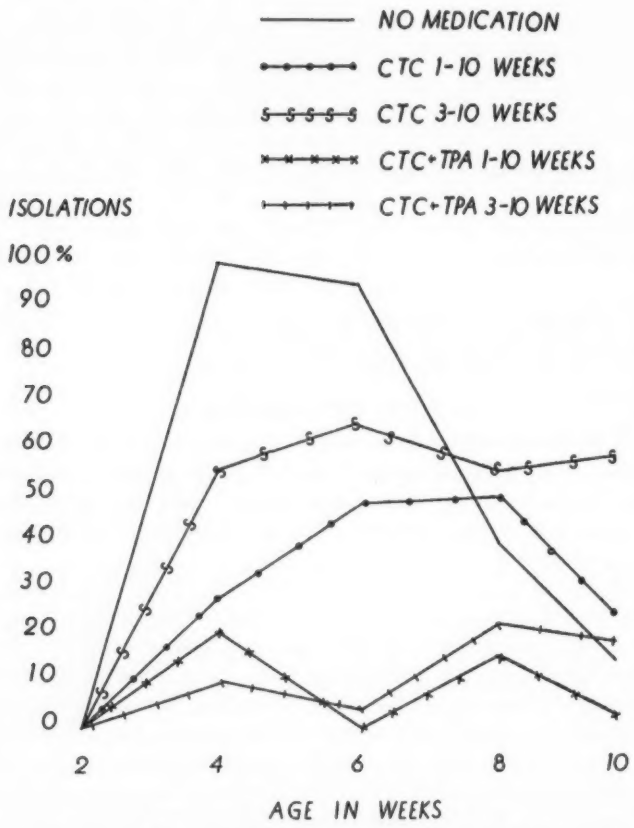

Fig. 1.....The percentage of isolations of MP from the trachea of non-medicated birds and from birds fed 400 grams of CTC with and without 0.5 percent TPA continuously from a day of age and from two weeks of age.

at four weeks increased the susceptibility of the birds to MP.

When the medications were started at the end of the fourth week and fed for the fifth and sixth weeks, the percentage of isolations was markedly reduced (figure 3, not cultured until the sixth week) and continued low as long as the medications were given. However, removal of the medi-

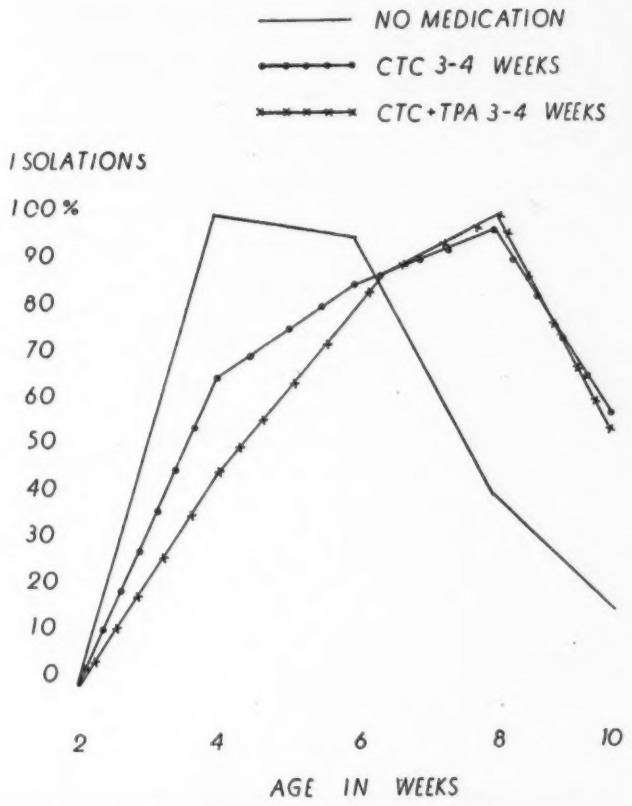

Fig. 2. The percentage of isolations of MP from the trachea of non-medicated birds and from birds fed 400 grams of CTC with and without 0.5 percent TPA for only the third and fourth weeks. (see figure 1 for the results of continuous medication from the second week.)

cation at the end of the sixth week resulted in a percentage isolation of MP that was slightly higher than when no antibiotic was given.

The medication at the end of the sixth week (figure 4, not cultured until the eighth week) resulted in a marked drop in the percentages of isolations. In the group of birds which

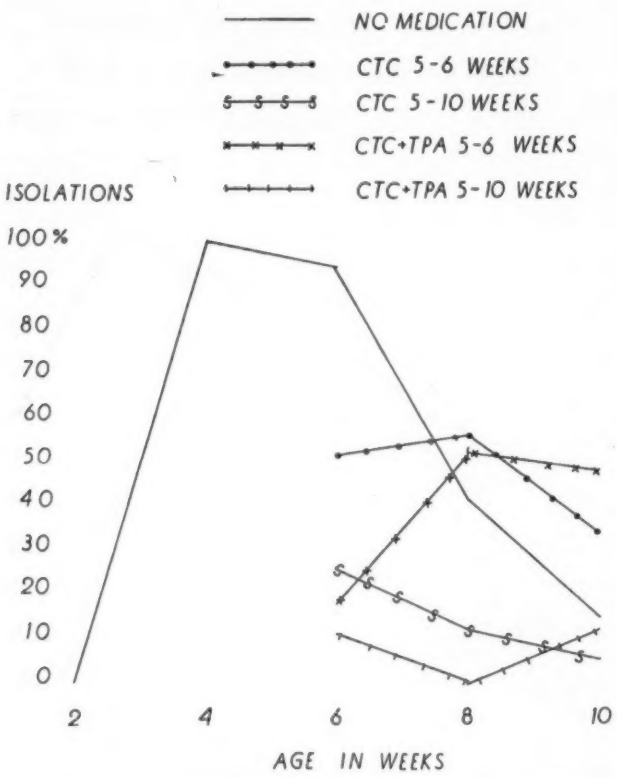

Fig. 3. The percentage of isolations of MP from the trachea of non-medicated birds and from birds fed 400 grams of CTC with and without 0.5 percent TPA for the fifth and sixth weeks and from the fourth week continuously.

was given CTC plus TPA, the percentage of isolation was zero at the eighth and tenth week.

Although additional experimentation is needed,under the conditions of this trial, medication during periods of active infection was of little value if given for a two-week period. It was interesting, however, that when CTC plus TPA was started at the end of the sixth week the percentage of isolations was reduced to zero in three of four pens. No increase

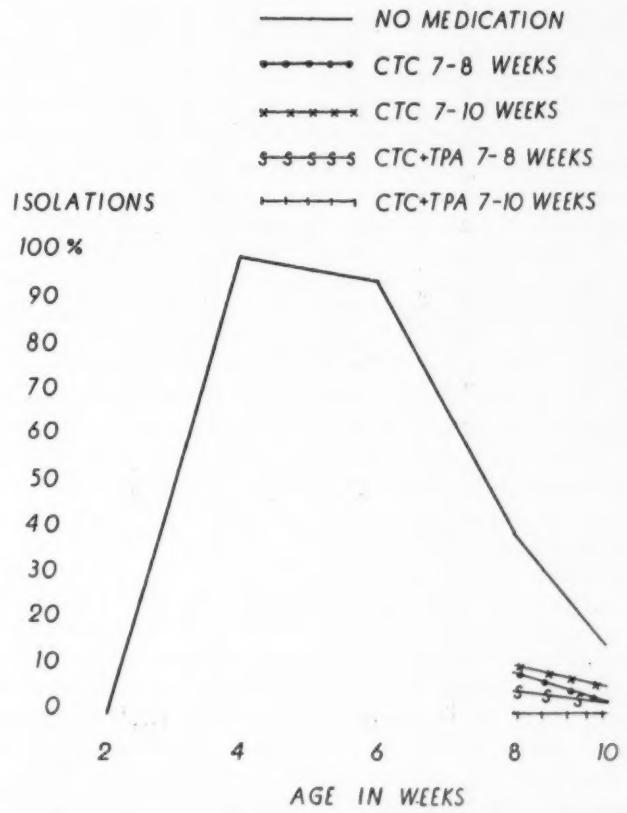

Fig. 4.....The percentage of isolations of MP from the trachea of non-medicated birds and from birds fed 400 grams of CTC with and without 0.5 percent TPA for the seventh and eighth weeks and from the sixth week continuously.

in the percentage of MP isolation occurred after the medication was removed.

The continuous medication with CTC reduced the percentage of isolations of MP as did the two week feeding period. Starting treatment 4 weeks after inoculation appeared to be the desirable period of medication for elimination of MP. Perhaps a longer period after infection would be desirable.

TABLE II

Results of duplicate pens at ten weeks of age when birds were fed CTC (400 grams per ton of feed) at various time intervals with and without 0.5 percent TPA

Duration of Treatment in weeks Weeks fed	0 0	2 3-4	2 5-6	2 7-8	4 7-10	6 5-10	8 3-10	10 1-10
			Chlortetracycline—No Terephthalic Acid					
Weight, lbs.	3.44	3.32	3.55	3.39	3.48	3.52	3.66	3.69
Feed conversion	2.59	2.77	2.71	2.77	2.76	2.75	2.62	2.67
Mortality %	3.0	3.0	3.5	2.0	3.5	1.0	2.0	1.0
			Chlortetracycline plus 0.5 Percent Terephthalic Acid					
Weight, lbs.	3.24	3.53	3.57	3.46	3.51	3.47	3.60	3.59
Feed conversion	3.02	2.68	2.71	2.63	2.79	2.83	2.71	2.81
Mortality %	2.0	0.5	1.0	3.00	0.5	1.5	1.0	3.0

An approach would be to infect day-old chicks, allow the infection to develop for six weeks, then medicate at high levels for 2 to 4 weeks. The birds should develop a high degree of immunity by this time and chances of reinfection following medication removal should be reduced.

The above procedure would be practical for replacement birds providing egg transmission is eliminated by this vaccination procedure. Since egg transmission is considered to be low, considerable investigation would be necessary to establish the value of this approach. Even though the MP was eliminated from the trachea, it is still possible that MP would be present in the air sacs or other organs. It may be possible to select pathogenic strains for this vaccination procedure that do not involve the air sacs. Many questions can be raised but the proof of any procedure must depend on further research. Culture procedure involving large numbers of birds are costly and time consuming. However, this appears to be the best method available for evaluating the results.

It should be pointed out that all birds in these trials were exposed to MP. Under conditions as they exist in the field, such high levels of antibiotics may be of value in preventing the spread of CRD.

The body weights, feed conversion, and mortality are shown in table 1. Analysis of variance established that the least significant difference for body weight was 0.279 pounds. The feeding of CTC for 1 to 10 and 3 to 10 weeks resulted in significantly greater weights than when no CTC was fed.

There was no significant difference in feed conversion among periods, but there was an interaction between treatment and periods. This means that the difference between treatments was not the same for all periods of treatment.

ACKNOWLEDGMENTS

The authors are grateful to the American Cyanamid Company for supplying the chlortetracycline, the E. I. DuPont De Nemours and Company, Incorporated for supplying the terephthalic acid, and Dr. R. S. Dunbar, Jr., Station Statistician for the statistical analysis.

SUMMARY

The feeding of high levels of chlortetracycline during periods of stress was of little value in controlling CRD. The feeding of CTC continuously did not eliminate infection, but when given 4 weeks after infection 400 grams CTC plus 0.5 percent TPA appeared to eliminate Mycoplasma.

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STUDIES ON PASTEURELLOSIS. IV. KILLED FOWL CHOLERA VACCINE ABSORBED ON ALUMINUM HYDROXIDE*

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THREE experiments are reported in this paper. Experiment A was a study to determine the efficacy of aluminum hydroxide gel as an adjuvant in killed fowl cholera vaccine. Expriment B was designed to determine (1) if this vaccine would stimulate and maintain a high degree of immunity for 52 weeks, and (2) if the stress of mixing vaccinated males from different flocks at time of challenge would affect their resistance to fowl cholera. Experiment C was a study to determine if birds could be immunized against fowl cholera and withstand exposure to virulent cultures of *Pasteurella multocida* while infected with fowl pox virus. A method for production of an effective water-in-oil emulsified fowl cholera veccine was presented in a previous paper.[1]

MATERIALS AND METHODS

Chickens. New Hampshire chickens from closed flocks and free from all infectious diseases, with the exception of 10 birds with fowl pox in experiment C, were used in these experiments. No chemotherapeutic agents, antibiotics, or other vaccines were used in raising these birds.

Preparation of vaccine. A culture of *P. multocida* was grown and harvested as previously described.[1] In experiment

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A, the bacterial concentration was adjusted to 25 × McFarland 1 density (approximately 3.25×10^{10} bacteria per ml). One part aluminum hydroxide gel* was mixed with 6 parts killed bacterial suspension in a Waring Blendor for 2 minutes. In experiments B and C, the bacterial concentration was adjusted to 10 × McFarland 1 density. Fifty ml of aluminum hydroxide gel was added to 500 ml of the killed bacterial suspension (1 part gel to 10 parts bacteria) and shaken in a 2-liter rubber-stoppered flask for 10 seconds 8 or 10 times during the day.

Vaccination and challenge. One dose of 0.5 ml of vaccine was injected subcutaneously in the midportion of the neck. The vaccine required frequent agitation to keep the adsorbed bacteria in suspension. The immunity of each bird was challenged as previously described[1] by swabbing the nasal cleft with a suspension of virulent *P. multocida* strain P-936. All birds that died were necropsied and cultured to confirm the diagnosis of fowl cholera, and moribund birds were killed and also necropsied and cultured. All survivors were observed for 10 or more weeks after they were exposed to *P. multocida.*

Experiment A. Eighty-six 17-week-old chickens were divided into two groups. One group of 51 birds (34 males and 17 females) was vaccinated and confined to finishing batteries in an isolation room. The other group (35 females) was held as unvaccinated, isolated controls in a 12 by 16 foot chicken house with a grass runway. Three lots of the vaccinated birds (16 males and four females, 17 males, one male and 13 females) and three lots of unvaccinated isolated controls (all females) 10, 10, and 15 were selected and their immunity challenged at 7, 16, and 36 weeks respectively postvaccination. The birds at the time of challenge were confined to batteries identical in type to those used for the vaccinated birds.

Experiment B. Two hundred and thirty-three birds were used in this experiment. Sixty-three 12-week-old males were vaccinated and placed with 24 unvaccinated contact

* Amphojel, Wyeth Laboratories, Inc., Philadelphia, Pa.

control males in two 20 by 20 foot masonry chicken houses. Ninety birds (84 females and six males) were also vaccinated at 12 weeks of age and placed with 16 (13 females and three males) unvaccinated contact controls in two masonry houses identical in type. Forty birds (28 females and 12 males) were raised in a separate isolation area as isolated controls.

At 16 weeks postvaccination, the two houses of vaccinated and contact control males were exposed to a virulent culture of *P. multocida;* the birds were stressed at time of exposure by redistributing the males between the two houses. At the same time, 13 females and seven males of the isolated controls were confined to a fattening battery in an isolation room and exposed to the virulent culture.

At 52 weeks postvaccination, the remaining two houses of vaccinated and contact control birds, which represented laying flocks, and the isolated controls were exposed to a virulent culture of *P. multocida.* The vaccinated and contact control birds were exposed to the culture in the same houses in which they were raised after vaccination. The isolated controls were confined to a fattening battery in a separate isolation room. The palatine cleft of each bird was swabbed, with cotton on a wood applicator, 20 weeks after they were exposed to *P. multocida.* The swabs were placed in tubes of 1 percent peptone broth. After incubating for 3 to 4 hours, the broth cultures were streaked on dextrose starch agar (Difco) plates. The plates were examined after 24 hours of incubation at 37 C for the presence of *P. multocida* colonies.

Experiment C. Forty 17-week-old pullets were used in this experiment. Ten birds infected with fowl pox and 20 birds free of disease were vaccinated and confined to different fattening batteries in the same room. Ten isolated controls were confined to a battery in a separate isolation room. At 2 weeks postvaccination, the immunity of each bird was challenged with a virulent culture of *P. multocida.* Forty-four sibs of the fowl pox birds were observed during the experiment.

RESULTS

The chickens suffered no apparent ill effects because of

vaccination. A few birds excreted greenish droppings for the first day or two after vaccination.

Experiment A. The results recorded in table 1 show that aluminum hydroxide gel was satisfactory when used as an adjuvant in the killed fowl cholera vaccine. All unvaccinated birds died within 3 days (average 1.5 days) after challenge, except one that died within 5 days. Two vaccinated birds died within each of the following intervals: 14 to 57 days after the 7-week challenge, 5 to 7 days after the 16-week challenge, and 3 to 11 days after the 36-week challenge.

Experiment B. Mortality figures in table 1 show that the vaccine did produce a high degree of immunity for at least 52 weeks in the two laying flocks. *P. multocida* was not recovered from the specimens taken from the palatine clefts of the laying flocks. However, 44 percent of the vaccinated males died when exposed to the virulent culture of *P. multocida* at 16 weeks postvaccination. When the social or peck order of the males was changed by mixing unacquainted males together at time of exposure, there was much fighting and bloodshed. The prostrated males were trodden by the more vigorous males.

The average time from the 16 weeks postvaccination challenge of vaccinated males until death was 13 days with a maximum of 46 days and a minimum of 1 day. The average time from the 52 weeks postvaccination challenge for the 12 vaccinated birds until death was 5.5 days with a maximum of 13, and a minimum of 1 day. The average time from challenge until death for all unvaccinated controls of both groups was 2 days.

Experiment C. Five of 10 vaccinated birds that had fowl pox at the time of vaccination and challenge died of fowl cholera, but only 1 of 20 vaccinated birds that were healthy at time of vaccination and challenge, died. All 10 unvaccinated isolated controls died of cholera. Forty-four sibs of the 10 pullets with fowl pox made an uneventful recovery from fowl pox.

DISCUSSION

Although the vaccine used in experiment A contained

TABLE I

Mortalities of chickens exposed to *Pasteurella multocida*

Chicken Groups	Weeks postvaccination challenge									
	2		7		16		36		52	
	D/C	%	D/C	%	D/C	%	D/C	%	D/C	%
Experiment A										
Vaccinated	—	—	2/20	10	2/17	11.8	2/14	14.3	—	—
Controls:										
Isolated	—	—	10/10	100	10/10	100	15/15	100	—	—
Experiment B										
Vaccinated*	—	—	—	—	28/63	44.4	—	—	—	—
Vaccinated	—	—	—	—	—	—	—	—	12/90	13.3
Controls:										
Isolated	—	—	—	—	20/20	100	—	—	20/20	100
Contact	—	—	—	—	24/24	100	—	—	15/16	93.8
Experiment C										
Vaccinated	1/20	5	—	—	—	—	—	—	—	—
Vaccinated**	5/10	50	—	—	—	—	—	—	—	—
Controls:										
Isolated	10/10	100	—	—	—	—	—	—	—	—

D = number died; C = number challenged; % = percent mortality
* Stressed by mixing unacquainted males at time of challenge
**Birds with fowl pox at time of vaccination and challenge

approximately two and one-half times more organisms than the vaccine used in experiments B and C, there was little difference in the efficiency of the two vaccines.

The contrast between the 12 percent death rate of the vaccinated males challenged at 16 weeks postvaccination in experiment A and the 44 percent death rate of the corresponding group of males in experiment B was because of mixing the males from different houses in experiment B at the time of exposure to *P. multocida*. Likewise, the stress of fowl pox at vaccination and challenge times probably accounted for the high death rate in experiment C. However, it is not known whether the fowl pox virus interfered with the production of fowl cholera antibodies or whether the added stress of the pox virus at time of exposure to *P. multocida* accounted for the higher rates.

The aluminum hydroxide adsorbed vaccine did not stimulate a degree of immunity higher than that produced by the water-in-oil emulsified vaccine.[1] However, it has the advantages of simplicity of production and ease of administration at low temperatures.

When a vaccine does not always give protection against fowl cholera, we must look for different immunogenic types of *P. multocida*. It was our opinion that only type 1 was necessary in the production of fowl cholera vaccines. Contrary to this , the authors have isolated at least two different immunogenic types from birds with fowl cholera. A vaccine made from one type will not protect against the other, but a bivalent vaccine made from the two types will protect against both. We also reported[1] that strains isolated from cattle would not produce fowl cholera. However, we have isolated a strain from the nose of a calf that will produce this disease. It was necessary to pass the strain through chickens several times in order to increase its virulence. Work is now in progress in this laboratory to find different immunogenic strains of *P. multocida*. The authors would appreciate receiving cultures of *Pasteurella* accompanied with a short history of each strain.

Probably one would not vaccinate birds which had an intercurrent disease, nor mix unacquainted males during a

disease outbreak. However, these experiments emphasize the role of good management in the control of disease.

SUMMARY AND CONCLUSIONS

As an extension of studies on the use of killed fowl cholera vaccine, data are reported on (1) the efficacy of aluminum hydroxide adsorbed vaccine (2) the effect of stress caused by changing the social or peck order of vaccinated males at time of exposure to *Pasteurella multocida*, and (3) the effect of a fowl pox at the time of vaccination, and exposure to virulent *P. multocida*.

An aluminum hydroxide adsorbed killed fowl cholera vaccine produced an effective immunity to fowl cholera for at least 52 weeks.

The mixing of unacquainted, vaccinated males at time of exposure to *P. multocida* considerably lowered their resistance to fowl cholera infection.

Birds with fowl pox at time of vaccination against and exposure to fowl cholera were more susceptible than birds that were free of fowl pox at time of vaccination and exposure.

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SURVIVAL OF MYCOPLASMA IN BROTH AND SEMI-SOLID MEDIA

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VARIOUS media and culturing techniques have been employed for the differentiation of avian Mycoplasma and bacterial L-forms. Cultivation of Mycoplasma in media containing reduced amounts of horse serum had resulted in an increased frequency in the recovery of bacteria.[5] In an attempt to duplicate these findings, several strains of Mycoplasma were grown in broth media. However, some of the cultures failed to grow when the concentration of horse serum was reduced much below 10 percent. Failure to survive in media with reduced amounts of horse serum made utilization of the technique impractical for studies on the possible reversion of these cultures to bacteria. Therefore, it was considered necessary to determine the ability of various strains of Mycoplasma to survive transfer in media with different concentrations of horse serum.

Domermuth and Gross[2] obtained good growth of *Mycoplasma gallisepticum* in semi-solid media containing bovine serum fraction (Difco). Similar results were obtained at this laboratory using semi-solid media with 10 percent horse serum. The relative value of semi-solid and broth media for the maintenance of Mycoplasma cultures has not been reported.

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The purpose of this study was to determine the effect of reduced concentrations of horse serum on the survival of Mycoplasma during transfer in broth and semi-solid media.

MATERIALS AND METHODS

Media. Beef heart infusion broth and agar, enriched with 1 percent proteose peptone no. 3 and 1 percent yeast hydrolysate were used as the base media. Semi-solid media were prepared by the addition of 0.2 percent agar to the basal broth media. Broth and semi-solid tubes had a volume of 3 ml of media and horse serum was added in amounts of 0.83, 0.15 and 0.3 ml which were designated as 1, 5, aond 10 percent, respectively. After addition of the horse serum, all tubes were incubated at 37 C for 48 hours before inoculation.

The agar medium was prepared in 100 ml amounts and stored at 4 C. When needed, this was melted, cooled to 45 C and 10 ml of horse serum added to each flask. The total of 110 ml was sufficient for approximately 15 petri dishes of 60 mm. This was designated as non-inhibitor agar. Inhibitor agar plates were prepared in the same manner except that thallium acetate and penicillin were added with the horse serum in amounts sufficient to give a final concentration of 1:4000 and 500 units per ml respectively.[3] Plates were incubated 48 hours at 37 C before inoculation.

Sufficient 0.1 N sodium hydroxide was added during preparation so that all media had a pH of 7.8 after sterilization at 121 C for 15 minutes.

Mycoplasma cultures. Sixteen strains of avian Mycoplasma were used. Ten strains failed to produce lesions when inoculated into the air sacs of chicks or turkey poults. Colonies on agar were the large "fried egg" type. These strains were considered to be of the "non-pathogenic" type or *Mycoplasma gallinarum.* The other six strains produced mild to severe lesions in the air sacs of turkey poults, usually produced smaller colonies on agar and were classified as the "pathogenic" type[1] or *Mycoplasma gallisepticum.* The culture number, type, and the original source of the individual strains are given in table 1.

TABLE I

Type and source of Mycoplasma strains used in survival study

Strain no.	Type	Original source
1	*M. gallinarum*	Strain J —University of Pennsylvania
4	*M. gallinarum*	Strain B-16 —Penn State University
12	*M. gallinarum*	Strain A-36 —University of California
14	*M. gallinarum*	Strain 91-R —Cornell University
18	*M. gallinarum*	Strain CRD-Swift —University of Rhode Island
19	*M. gallinarum*	Case 2682 —Calif. Dept. of Agriculture
32	*M. galilnarum*	Case PS 2510 —Penn State University
34	*M. gallinarum*	Strain 38 —University of Delaware
47	*M. gallinarum*	Strain #2906 —University of Minnesota
48	*M. gallinarum*	Strain #2907 —University of Minnesota
50	*M. gallisepticum*	Strain S6 —Cornell University
52	*M. gallisepticum*	Strain VB —Cornell University
53	*M. gallisepticum*	Strain A-5969 —University of Connecticut
54	*M. gallisepticum*	Strain S6G6 —University of California
55	*M. gallisepticum*	Winchester Strain —Virginia Polytechnic Institute
57	*M. gallisepticum*	Strain Md. 2 —University of Maryland

Transfer procedures. Cultures were transferred twice weekly for a total of 20 passages. Each strain was carried in four broth and four semi-solid media. Three of the broth and three semi-solid groups had horse serum present at all times at levels of 1, 5, and 10 percent, while in the fourth group, horse serum was omitted from alternate passages. In these two latter groups, the seed cultures were first transferred to media without horse serum. For the second transfer, 10 percent horse serum was present while the third was again without serum. Alternate passage with and without horse serum continued for 20 transfers or as long as the individual strain survived.

The method of transfer differed for the two kinds of media. Broth tubes were tapped gently to resuspend any of the organisms that had settled to the bottom of the tube. A pipette was then inserted until the tip touched the bottom of the tube and 0.3 ml withdrawn. In semi-solid, the growth was usually in a narrow band near the top of the medium. Therefore, the pipette was inserted to just below the surface and the 0.3 ml secured for transfer. The 0.3 ml, which was approximately 10 percent of the total culture, was used routinely as the transfer volume.

Each of the strains had been through three passages in eggs just prior to checking for pathogenicity in chicks and turkey poults. Seed cultures were prepared in 10 percent horse serum broth using the same source of inocula (harvests from the 3rd egg passage) as had been used for the birds. After 72 hours incubation, the initial transfers were made into the eight different media. At the same time a portion of the seed culture was titrated[6] in serial 10-fold dilutions in broth and 0.1 ml of each dilution immediately plated. Plates were examined after 72 hours incubation and the end-points were recorded as the highest dilution showing 5 or more colonies.

Survival of Mycoplasma was determined by plating after 4, 8, 12, 16, and 20 passages. Heavy growth in broth and semi-solid tubes could be easily detected. However, some tubes which appeared negative by gross examination, pro-

duced abundant growth on plates.. Therefore, direct readings from passage tubes were not attempted.

When bacteria were detected, transfers were stopped, and the culture streaked on non-inhibitor agar. After 48 hours incubation, single colonies were transferred to 10 percent horse serum broth which was incubated 24 hours and then streaked on inhibitor and non-inhibitor agar. Plates were checked daily through 7 days for Mycoplasma, bacterial growth or both for the possible detection of L-form reversion.[3]

RESULTS AND DISCUSSION

The original titers of the various strains ranged from 10^{-4} to 10^{-8}. There was no apparent relationship, however, between the magnitude of the original titer and subsequent survival in the different media.

All strains in each medium had been plated after 4, 8, 12, 16, and 20 passages. The last platings to show growth with each strain in the different media are listed in table 2. Broth with 10 percent horse serum was the only one that supported growth of all strains for the full 20 passages. Elimination of horse serum from every other passage did not alter the survival of the *M. gallinarum* strains except with strain 14 which showed bacteria at the 14th passage. However, three of the *M. gallisepticum* strains apparently failed to grow in the first passage which contained no horse serum and were negative on the first plating (passage 4). Reduction of horse serum to 5 or 1 percent still supported growth of most of the strains but some became gradually weaker and were negative after 12 or 16 passages. Strain 52 apparently required a relatively high concentration of horse serum since it failed to grow in 1 percent broth and survived only to the 4th passage in 5 percent broth. Strain 32 grew well in broth with 1 percent horse serum and its failure to survive with 5 percent horse serum could not be explained.

In semi-solid media, 10 percent horse serum was again the most satisfactory with six strains surviving through 20 passages. One strain, number 34 also carried through all 20 passages in 5 percent and alternate 0-10 percent media. Most

TABLE II

Survival of *Mycoplasma* in broth and semi-solid media

Strain no.	Orig. titer	Broth Serum concentrations 1%	5%	10%	Alt. 0-10%	Ave.**	Semi-solid Serum concentrations 1%	5%	10%	Alt. 0-10%	Ave.
M. gallinarum											
1	6§	20§§	20	20	20	20	12	12	20	16	15
4	6	20	20	20	20	20	12	12	16	B (12)	13.3
12	4	16	20	20	20	19	12	12	12	8	11
14	4	16	20	20	*B(14)	18.7	12	12	B (11)	12	12
18	4	16	16	20	20	18	12	12	20	8	13
19	8	20	20	20	20	20	B (13)	16	20	B (7)	18
32	6	20	4	20	20	16	12	16	20	B (9)	16
34	6	20	20	20	20	20	12	20	20	20	18
47	5	20	20	20	20	20	B (7)	8	8	B (8)	8
48	6	B (14)	20	20	20	20	B (6)	8	8	8	8
M. gallisepticum											
50	6	20	20	20	0	15	B (16)	12	12	12	12
52	4	0	4	20	0	6	B (7)	12	12	8	10.7
53	5	12	20	20	20	18	8	8	B (11)	12	9.7
54	5	20	B (15)	20	20	20	8	B (11)	20	B (10)	14
55	5	20	16	20	0	14	B (11)	B (6)	16	B (9)	16
57	4	16	20	20	20	19	8	8	12	12	10

*B —Passage when bacteria detected in broth
§ —Value of the negative exponent of the highest log dilution showing 5 or more colonies e.g. 6 indicates log 10^{-6} dilution.
**Ave.—Average number of passages survived - cultures showing bacteria not included in the calculation.
§§ —Number indicates highest passage positive for Mycoplasma on plates.

of the other strains survived only 8 to 12 passages or showed the presence of bacteria.

Broth was much better than semi-solid with respect to the number of strains that survived all 20 passages. Without considering the concentration of horse serum, 48 of the 64 broth cultures, and only eight of the 64 semi-solid cultures were alive at termination of the experiment. Growth in the semi-solid tubes was easily detected in early passages. Dense bands of the organisms were evident near the top of the medium. As the number of passages increased, the band became less pronounced and what appeared to be individual colonies could be seen. In some cases the number of colonies gradually decreased, but most frequently a culture that was growing well would, for no obvious reason, fail to grow on transfer.

The average survival rates were calculated for each type of medium and are given in table 3. The cultures that showed bacteria were not included in these calculations. Except for broth with 10 percent horse serum, where all cultures survived the full 20 passages, the survival rate was higher for *M. gallinarum* than for *M. gallisepticum*. Also, the survival rate increased with increasing amounts of horse serum in both broth and semi-solid.

TABLE III

Average survival rates of Mycoplasma in broth and semi-solid media

	Transfer media							
	Broth				Semi-solid			
	Serum concentrations				Serum concentrations			
Type	1%	5%	10%	Alt. 0-10%	1%	5%	10%	Alt. 0-10%
M. gallinarum	18.7*	18.0	20.0	18.0	12.0	12.8	17.1	12
M. gallisepticum	14.7	16.0	20.0	10.0	8.0	10.0	14.4	11
All strains	17.1	17.3	20.0	16.0	10.8	12.0	16.1	11.6

* Ave. number of passages survival - cultures showing bacteria not included in the calculation.

The frequency of the recovery of bacteria was much greater in semi-solid media than in broth. In semi-solid media, 18 of the 64 cultures showed bacteria while in broth only three of the 64 showed bacteria. If the number of cultures showing bacteria were a measure of reversion from bacterial

L-forms, the semi-solid media would be considered much the best. However, when the bacteria were plated in inhibitor and non-inhibitor agar,[3] there was no evidence of reversion and the bacteria had to be considered as contaminants. Since the presence of the bacteria could not be accounted for as reversions from L-forms, the reason for the increased frequency of bacteria in semi-solid media could not be determined. All 21 of the bacterial cultures were gram positive cocci and identified as Micrococci.

SUMMARY

Ten strains of *M. gallinarum* and six strains of *M. gallisepticum* were transferred in broth and semi-solid media containing various amounts of horse serum. Broth with 10 percent horse serum was the only medium which supported growth of all cultures for the full 20 passages.

The average rate of survival decreased with the reduced concentration of horse serum, especially with the semi-solid media. The average survival of *M.gallinarum* was consistently better than for *M. gallisepticum*.

Bacteria, identified as Micrococci, were recovered from 16 of 64 semi-solid transfer cultures but from only three of 64 broth cultures. Testing of the bacteria on differential media failed to show evidence of reversion from bacterial L-forms and the significance of this difference could not be determined.

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STUDIES WITH EASTERN ENCEPHALITIS IN WHITE PEKIN DUCKLINGS

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THE duck was found to be one of the many domestic animals susceptible to intracerebral inoculation of eastern encephalitis (EE) virus by Giltner and Shahan.[2] Howitt[3] noted that Pekin ducks usually succumbed to intracerebral inoculation of western virus, and Remlinger and Bailly[5] indicated that the mallard duck was susceptible. The first case of EE in White Pekin ducklings on Long Island was reported by Dougherty *et al.*[1] This paper presents some of the data gained from preliminary studies of the action of the EE virus upon ducklings.

MATERIALS AND METHODS

The virus used in these experiments was isolated from the brain and spleen of a 2-week-old duckling (accession #5536). The organs were ground in Ten Broeck grinders with 1.0 ml tryptone broth containing 10,000 units penicillin and 10 mg streptomycin. The material was centrifuged and 0.2 ml of the supernatant was inoculated into the yolk sacs of 6-day-old embryonated chick eggs. The first deaths occurred in 18 hours. Six passages of the virus were made in chick embryos and the harvested yolk material was used in the experiment. The virus titer was $10^{-7.2}$ in chick embryos and

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$10^{-7.0}$ in 3-week-old white mice. In duckling kidney monocell layers,[4] the titer was $10^{-5.6}$.

The ducklings used were from two sources. One was an upstate New York farm 450 miles from Long Island, and the other source was the experimental farm at Eastport, L. I. The intramuscular route (IM) of inoculation was chosen because previous experiments had shown it to be the most satisfactory.[1]

Immune guinea pig sera for western and eastern encephalitis were used for the neutralization studies. One-half ml of serum was mixed with 500,000 embryo lethal doses (ELD) of virus and held for 1 hour at room temperature before the mixture was injected IM into the birds.

RESULTS

The results of the neutralization of duck origin EE virus by eastern and western immune sera are summarized in table 1.

TABLE I

Neutralization in susceptible day old ducklings of EE virus (500,000 ELD's) by immune sera

Type of sera	Duckling number 1	2	3	4	5	6
EE serum + virus	dead 16 hrs.*	dead 30 hrs.	dead 30 hrs.	dead 30 hrs.	Alive	Alive
WE serum + virus	dead 25 hrs.	dead 30 hrs.	dead 30 hrs.	dead 30 hrs.	dead 30 hrs.	dead 30 hrs.
Virus	dead 2 hrs.	dead 23 hrs.	dead 25 hrs.	dead 25 hrs.	dead 30 hrs.	dead 30 hrs.
Contact controls	dead 30 hrs.	Alive	Alive	Alive	Alive	Alive

* Time post challenge

These ducklings were from the upstate New York farm; it can be noted in the table that one of the contact controls died in 30 hours, and EE virus was recovered from the brain. The experiment had been carried out in a Rockefeller type isolation building which was free of mosquitoes and flies.

One-week-old ducklings from the Long Island experimental farm were inoculated intramuscularly with 500,000 ELD of the virus and bled hourly for the first 6 hours and

TABLE II

Virus levels in the blood of 1-week-old ducklings inoculated intramuscularly with EE virus

Duck	Time in hours																	
	0	1	2	3	4	5	6	12	18	24	30	36	42	48	54	66	72	80
1	*0.0	5.0	4.0	5.0	6.0	5.2	6.0	2.8	4.3	3.8	3.8	2.3	1.8	2.0	0.0	0.0	0.0	0.0
2	0.0	2.8	4.0	7.0	6.0	4.7	6.0	6.0	6.0	3.2	2.4	2.5	1.0	0.0	0.0	0.0	0.0	0.0
3	0.0	5.0	2.7	4.3	6.0	5.8	0.0	5.0	4.4	0.0	0.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0
4	0.0	2.9	2.8	4.7	5.0	5.5	6.0	4.6	4.2	6.0	5.0	0.0	0.0	0.0	0.0	—	0.0	—
5	0.0	4.0	2.7	5.4	6.0	6.6	3.7	5.0	4.4	6.0	6.0	4.0	2.5	2.0	—	—	—	—
aver-age	0.0	3.9	3.2	5.3	6.0	5.5	4.3	4.7	4.7	4.2	3.6	2.6	1.1	1.0	0.0	0.0	0.0	0.0

* Virus titer.

thereafter at 6 hour intervals through 80 hours post-inoculation. The serum was titrated in 6-day-old embryonated chick eggs. At 1 hour the embryo LD_{50} of the blood virus level was 3.9* (approximately the dilution of the inoculum in the body fluids). The peak was reached in 4 hours as shown in table 2. No virus was detected after 48 hours.

Six old breeders from the laboratory flock were test bled prior to IM inoculation with 500,000 ELD of the virus. Neither virus nor SN antibodies were present. The birds were bled at 4, 24, 48, 72, and 96 hours. The virus blood levels are given in table 3.

TABLE III

Virus and SN levels in the blood of old breeders inoculated intramuscularly with EE virus

			Time in hours					
Duck	Sn titer	Pre-inoc	4	24	48	72	96	SN titer pools at 96 hours
1	0.0	0.0	2.3	2.7	0.0	0.0	0.0	
2	0.0	0.0	4.0	2.5	0.0	0.0	0.0	3.2
3	0.0	0.0	3.0	2.4	0.0	0.0	0.0	
4	0.0	0.0	1.6	2.6	2.0	0.0	0.0	
5	0.0	0.0	1.6	2.4	0.2	0.0	0.0	4.5
6	0.0	0.0	1.6	2.5	0.7	0.0	0.0	
Average	0.0	0.0	2.4	2.5	0.5	0.0	0.0	3.8

The greatest concentration of virus in the samples was at 4 hours in two birds and 24 hours in four birds. No virus was present after 48 hours.

An experiment was designed to show the comparative virus levels in 1-week-old ducklings and 1-week-old chickens. The birds were divided into two groups. One group, ducks and chickens, received the high level of virus, 500,000 ELD, and the second group received a low level, 100 ELD. Two chickens and two ducks in each group were bled at 1, 4, 8, 24, 48, 72, 96 and 120 hours. At 8 hours the average titer in the ducks was 4.6 and 4.8 in the chickens. At 72 hours the duck average was 0.0 and the chickens average was 0.1 (table 4.) There was no virus present in the chickens 24 hours later.

* The logarithmic LD_{50} is expressed as the exponent of the reciprocal of the end point dilution throughout this paper.

TABLE IV

Blood virus titers of 1-week-old ducklings and chicks infected with 100 and 500,000 ELD_{50} EE virus

	Time in hours							
	1	4	8	24	48	72	96	120
Inoculum 100 ELD_{50} EE virus								
Duck 1	0	0.5	4.6	3.5	0	0	0	0
Duck 2	0	2.4	4.6	5.2	0	0	0	0
Average	0	2.4	4.6	4.4	0	0	0	0
Chick 1	0	2.0	5.3	4.9	1.2	0.2	0	0
Chick 2	0	2.0	4.3	4.0	1.4	0	0	0
Average	0	2.0	4.8	4.4	1.3	0.1	0	0
Inoculum 500,000 ELD_{50} EE virus								
Duck 1	2.8	4.3	5.4	4.2	0	0	0	0
Duck 2	1.9	5.5	7.4	4.0	0	0	0	0
Average	2.4	4.9	6.4	4.1	0	0	0	0
Chick 1	3.8	3.6	6.1	5.5	2.5	0	0	0
Chick 2	3.3	5.4	6.8	2.9	2.0	0	0	0
Average	3.6	4.5	6.4	4.2	2.2	0	0	0

The peak was reached at 8 hours in both ducks and chickens receiving 500,000 ELD of virus. The level in both was 6.4, and both groups fell rapidly after that. The ducks were negative at 48 hours, but the level in the chickens was 2.2. There was no detectable virus in the chickens at 72 hours. These results are shown in table 4.

SUMMARY

There was neutralization of the duck origin EE virus when mixed with eastern encephalitis immune guinea pig serum and inoculated into 2 day old ducklings. No neutralization occurred when western encephalitis serum was used.

The blood virus levels reached a peak from 4 to 8 hours in 1-week-old ducklings and fell to 0 from 48 to 54 hours after injection of either 100 ELD of virus or 500,000 ELD of virus. Approximately the same blood virus levels were reached in 1-week-old chickens in 8 hours, but the titers fell to 0 in 72 hours.

The virus level rose only to 2.5 in 24 hours in breeder ducks and dropped to 0 in 72 hours.

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FURTHER STUDIES ON THE CAUSATIVE ORGANISM OF ULCERATIVE ENTERITIS*

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SINCE our original report describing the isolation of a bacterium from chickens capable of producing ulcerative enteritis in quail, (*Colinus virginianus*), subsequent studies have been made of this bacterium. In addition, investigations have been made of field outbreaks of ulcerative enteritis occurring in quail, turkeys, and chickens.

The chickens were from 5 to 10 weeks old and the poults were 4 to 7 weeks. Most of the cases of ulcerative enteritis in chickens and turkeys were preceded or accompanied by coccidiosis. Many of the cases were receiving sulfonamid medication. The most frequent sites of lesions in chickens were the intestine and ceca. The lesions consisted of small,

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hemorrhagic areas in the early stages with the gradual development of ulcerations in the mucosal surface. Perforation of the ulcers and peritonitis occurred in advanced cases. Occasionally, the mucosal surface consisted of a necrotic, diptheritic membrane. Although not always present, liver lesions consisted of focal, necrotic areas, irregular mottled areas, dark yellow focal areas surrounded by a light yellow halo and large, irregular, yellow necrotic areas particularly on the edges of the liver (figures 1, 2). Enlarged, hemor-

Fig. 1. Necrotic foci in the liver of a chicken with ulcerative enteritis.

rhagic spleens were frequently present (figure 2). A mortality of 1 to 5 percent was reported in these cases.

In poults, intestinal lesions were either crater-like, circumscribed ulcers or a brownish, necrotic, diphtheritic membrane occupying the middle third of the intestine (figure 3). This diphtheritic membrane in poults appeared similar to the lesion produced by *Eimeria brunetti* infection in chickens. Focal, yellow, necrotic areas were occasionally found in the liver. Stained smears of the necrotic foci occurring in livers

from quail, chickens, and poults revealed Gram positive rods, free spores and rods with subterminal spores.

EXPERIMENTAL

Isolations of the organisms. A total of 20 different isolations of the bacterium were made from quail, chickens, and turkeys infected with ulcerative enteritis. Isolations were made from quail blood, liver, intestine, and intramuscular fluid. All isolations with the exception of three were made by the inoculation of 5 to 6 day embryos. Following intraperitoneal injection of quail, three isolations were made by inoculating pieces of quail livers into thioglycollate. Isolations made by inoculation of embryos are listed in table 1.

Fig. 2. Large, irregular, necrotic areas (arrows) on the edges of a liver from a chicken with ulcerative enteritis. Note enlarged hemorrhagic spleen.

Cultural studies. Growth under normal atmospheric conditions was established in thioglycollate medium enriched with 3 to 10 percent horse serum or defibrinated horse blood. This growth occurred as a wool pack in the lower two-thirds of the tube after adaptation. The inocula for thioglycollate were yolk cultures, yolk agar cultures, and liver tissue from

quail which died following intraperitoneal injection. After eight to ten serial passages in thioglycollate, growth was established on yolk agar, PPLO agar with 3 percent serum fraction and 5 percent horse blood agar incubated in 10 percent CO_2, 63 percent methane, and 27 percent air. Initially, light growth occurred in 48 hours as discrete, smooth, raised, white, circular colonies 1 to 2 mm in size. After adaptation, the growth was not as moist and became confluent. Growth was also obtained when yolk cultures were seeded on yolk agar and blood agar in the same mixture of gases. However, cultures on agar seeded with yolk inoculum were difficult to

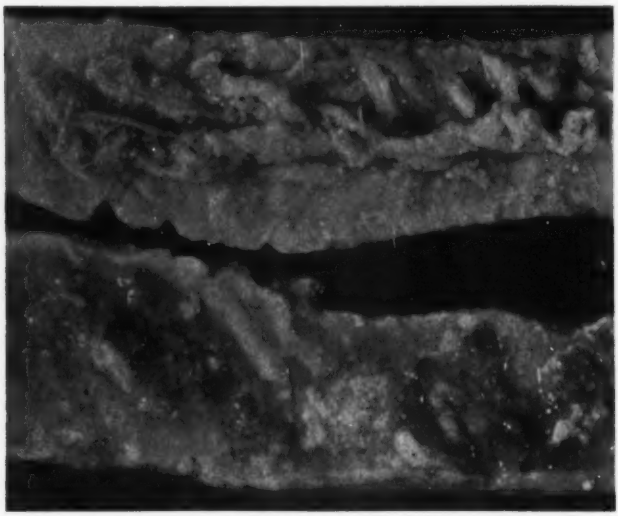

Fig. 3. Necrosis and sloughing of the intestinal mucosa of a poult with ulcerative enteritis.

maintain serially and usually failed to grow after several passages.

Growth was established on Dorset's egg slants without glycerol and on Loeffler's serum agar slants inoculated with thioglycollate cultures. Consistent fermentation results were not obtained in carbohydrate media. The use of a staphy-

lococcus streak on blood agar with or without reduced oxygen tension did not enhance growth.

It was noted that variations in morphology occurred with continued passage in artificial media. In addition to the rod forms, coccoid forms and chains were sometimes seeen. However, even after 14 serial passages in embryos, the organism still retained its rod morphology.

Spores were demonstrated in growth on blood agar, in thioglycollate cultures and in yolk from 7 to 9 day embryos. Spores were more numerous in early embryo passages. Spores could not always be demonstrated in cultures on artificial media.

TABLE I

Isolations of the ulcerative enteritis bacterium

Source of inoculum	Tissue cultured	Medium inoculated
Quail fed infected quail intestine	Blood	Embryo yolk sac
Quail fed yolk from second embryo passage	Liver	Embryo yolk sac
Quail fed yolk from embryo inoculated with 5th passage thioglycollate culture	Liver	Embryo yolk sac
Quail fed chicken intestine with ulcerative enteritis	Liver	Embryo yolk sac
Quail fed intestinal suspension	Liver	Embryo yolk sac
Quail fed intestinal suspension	Liver	Embryo yolk sac
Quail natural infection	Liver heated 60 C 15 minutes	Embryo yolk sac
Quail natural infection	Liver heated 60 C 15 minutes	Embryo yolk sac
Quail fed infected quail intestine	Liver	Embryo yolk sac
Quail injected intramuscularly with yolk culture	Pectoral muscle fluid	Embryo yolk sac
Quail natural infection	Intestine heated 70 C 10 minutes	Embryo yolk sac
Quail injected intraperitoneally with yolk culture	Liver	Embryo yolk sac
Quail natural infection	Liver heated 70 C 10 minutes	Embryo yolk sac
Quail natural infection	Liver heated 70 C 20 minutes	Embryo yolk sac
Chicken	Liver heated 70 C 60 minutes	Embryo yolk sac
Chicken	Liver heated 80 C 20 minutes	Embryo yolk sac
Turkey	Liver heated 70 C 45 minutes	Embryo yolk sac

Transmission studies. Quail were readily infected by oral inoculation of intestinal suspensions taken from field

cases of ulcerative enteritis occurring in poults, chickens, and quail. Death usually occurred within 3 to 12 days with intestinal and liver lesions identical to those seen in field cases. Quail were also infected by feeding necrotic livers taken from chickens and turkeys affected with ulcerative enteritis.

Yolk cultures were pathogenic for quail when administered by the oral, intramuscular and intraperitoneal routes. Death usually occurred within 1 to 3 days following intramuscular and intraperitoneal inoculation and within 3 to 12 days following oral inoculation. A white area occurred at the site of intramuscular injection and there was increased fluid between the muscle layers. If death occurred early, the intestinal lesions produced by intraperitoneal or intramuscular injection were either a markedly reddened mucosa or newly formed, acutely inflamed ulcers and if death was delayed several days, the ulcers became necrotic. The livers had a mottled appearance. Birds that died after feeding of yolk cultures had intestinal and cecal ulcers that varied in number and severity. In some cases, perforation accompanied by peritonitis occurred. Enlarged, hemorrhagic spleens were often present. The livers

TABLE II

Effect of storage, heating and *in vitro* cultivation on the pathogenicity of yolk inoculum for quail

	Treatment of inoculum			
Inoculum	Duration	Temperature	Subsequent cultivation*	Pathogenicity for quail
Yolk			5 serial embryo passages	+
Yolk			4 and 5 thioglycollate passages followed by embryo yolk sac	+
Quail intestine	5 months	20 C		+
1st passage yolk	14 months	20		+
4th passage yolk	12 months	20 C	embryo yolk sac	+
1st passage yolk	3 hours	70	embryo yolk sac	+
Yolk	10 minutes	80 C	yolk sac—80 C 1 hour—embryo yolk sac	
Yolk	15 minutes	80 C	embryo yolk sac	+
Yolk	3 minutes	100 C	embryo yolk sac	+

* Cultures in embryos resulted in death of the embryos

were mottled and often contained 1 to 2 mm gray, necrotic areas or larger, irregular yellow, necrotic areas. A smear of the necrotic liver lesions revealed Gram positive rods, free spores, and rods with subterminal spores.

Quail did not become infected with any cultures grown on artificial media. However, cultures passed four and five times in thioglycollate followed by one embryo passage were pathogenic when fed to quail. Other inocula were subjected to various treatments and still retained their pathogenicity (table 2).

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SUMMARY

Naturally occurring outbreaks of ulcerative enteritis in chickens, turkeys, and quail have been encountered. From these cases a Gram positive, spore forming rod was isolated in embryos by inoculation of liver or intestinal suspensions. Some of these inocula were heated for varying periods to a maximum of 70 C for one hour prior to embryo inoculation. Growth of the bacillus was established in thioglycollate media enriched with 3 to 10 percent horse serum. Initially, growth was obtained on solid media incubated under reduced oxygen tension. Subsequent growth was obtained under aerobic conditions on yolk agar, PPLO agar with 3 percent serum fraction, 5 percent horse blood agar, Loeffler's serum agar slants and Dorset's egg media. Cultures grown on artificial media lost their pathogenicity for quail.

The organisms isolated from quail, turkeys and chickens possessed similar characteristics and produced lesions typical

of ulcerative enteritis when yolk sac cultures were fed to quail. The organism was reisolated from quail which died following oral administration of yolk cultures. The bacterium was also pathogenic for quail by the intramuscular and intraperitoneal routes. Yolk cultures heated at different temperatures up to 80 C for varying periods up to one hour remained pathogenic for embryos. Yolk cultures remained pathogenic for quail after storage at 20 C for 12 months.

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ANTIBIOTIC RESISTANCE AND MUTATION RATES OF MYCOPLASMA*

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ANTIBIOTIC-RESISTANT strains of Mycoplasma (avian pleuropneumonia-like organisms, PPLO) have been selected by *in vitro*[3] and *in vivo*[4,8] methods. The *in vivo* studies indicate that PPLO rapidly develop resistance to streptomycin, that resistance to the tetracycline group of antibiotics is developed only with comparative difficulty, and that resistance to erythromycin is not developed. The last conclusion probably was due to the fact that the particular test strain of PPLO observed had not been sensitive to erythromycin originally. The *in vitro* study indicates that PPLO resistance to streptomycin increases in the one-step manner characteristic of

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bacteria. Mutation rates to increased resistance were not calculated in any of these studies.

Since complete information is not available concerning the way in which and the rate at which PPLO mutate to increased antibiotic resistance, it seemed desirable to continue studies to provide more information of this type. The purpose of this paper is to report the results of such a study.

MATERIALS AND METHODS

The Winchester (W) PPLO strain was used as the test strain in this study. The W strain was ideal for a study of this type because it was isolated in 1950 at this laboratory[5] before the general use of antibiotics, and has been carried *in ovo* without exposure to antibiotics since that time. It has also retained its original high degree of pathogenicity and is regarded as a typical pathogenic avian PPLO.[7] The strain used in this study was freshly isolated from an *in ovo* stock culture and propagated in media* for from 22 to 35 passages before being used.

The antibiotics used in this experiment were dihydrostreptomycin sulfate, erythromycin, tetracycline hydrochloride and chloromycetin. Media were prepared either with no antibiotics, or by the addition of antibiotic sufficient to give post inoculation concentrations of 0.1, 1.0, 10, 100, 1,000, or 10,000 microgram/ml of medium.** Each of the tubes thus prepared were inoculated with 1.0 ml of inoculum making a total of 10 ml per tube. The inocula were prepared by making 10 fold dilutions, ranging from undiluted through 10^{-10}, of a 3 day broth culture of the W strain. These cultures were incubated at 37 C for one month and observed twice weekly for presence or absence of visible turbidity throughout that period of time. The cultures which had developed from the smallest number of PPLO in each concentration of each of the antibiotics were subcultured and each subculture was again prepared and observed as above.

* Difco PPLO broth without crystal violet, 1% Difco PPLO serum fraction, 1/4000 parts thallium acetate, 1,000 units penicillin per ml, final pH adjusted to 7.8.

** Difco PPLO broth without crystal violet, 1% Difco PPLO serum fraction, 1/4000 parts thallium acetate, adjusted to a final pH of 7.8 after the addition of antibiotic.

RESULTS

Erythromycin (*table 1*). One in 10^4 to one in 10^6 (mutation rate = number of resistant organisms/number of normal organisms[9]) viable PPLO units which had not been previously exposed to antibiotics were found to be resistant to low levels (0.1 and 1.0 microgram/ml) of the antibiotic.

The cultures which had been exposed to 0.1 (tube b, table 1) and 1.0 (tube c, table 1) micrograms/ml of erythromycin grew in 10 micrograms/ml (the culture from tube b) and 100 micrograms/ml (the culture from tube c) respectively. Thus, in both instances, one in 10^8 viable PPLO units were capable of growing in an antibiotic concentration 100 times greater than they were previously capable of and thus exhibited a distinct rapid "step-wise" mutation to increased resistance.

The fact that the concentration of viable PPLO units, 10^5 and 10^7/ml in the unexposed culture and in the unexposed culture from tube a, respectively, was less than the normal maximum of 10^8 to 10^9 ml which is usually obtained for this type of culture, together with the observation that smaller mutation rates are obtained when a greater number of viable units are assayed (both here and following), suggests that mutation rates less than 1 in 10^8 are artifacts produced by the clumping of viable cells. The formation of viable units thus would reduce the apparent number of normal cells, the number of mutant cells assayed would remain unaffected, thus causing the apparent mutation rate to be greater.

Streptomycin (*table 2*). One in 10^6 viable PPLO units which had not been previously exposed to antibiotics were found to be resistant to as much as 10,000 micrograms/ml of streptomycin. Again, as with erythromycin, the concentration of viable PPLO units in these cultures was somewhat less than the normal maximum; thus, the mutation rates calculated for these two cultures are also greater than might be expected if a larger number of viable units had been assayed. The cultures which had been exposed to 0.1 (tube b) and 1.0 (tube c) micrograms/ml of streptomycin had not changed their degree of resistance to the antibiotic following these single exposures

TABLE I

The *in vitro** resistance and mutation rates of PPLO to erythromycin

Prior exposure to erythromycin	Micrograms/ml erythromycin	Mutation rates	Days post inoculation: 3	6	10	13	17	20	24	27	31
			Greatest inoculum dilution which initiated growth								
Unexposed	0		2**	5	5	5(a)	5	5	5	5	5
	0.1	1/10^{4}***	1	1	1(b)	1	1	1	1	1	1
	1.0								1	1	1(c)
	10.0										
Unexposed, subcultured from tube (a)	0		5	6	6	6	7	7	7	7	7
	0.1	1/10^{6}***		1	1	1	1	1	1	1	1
	1.0										
Exposed, subcultured from tube (b)	0		8	9	9	9	9	9	9	9	9
	0.1		2	2	3	3	3	4	4	4	4
	1.0	1/10^{8}	1	1	1	1	1	1	1	1	1
	10.0							1	1	1	1
	100.0										
Exposed, subcultured from tube (c)	0		6	9	9	9	9	6	9	9	9
	0.1		6	8	8	8	8	8	8	8	8
	1.0	1/10^{8}	5	7	9	9	9	9	9	9	9
	10.0		1	1	1	1	1	1	1	1	1
	100.0			1	1	1	1	1	1	1	1
	1000.0										

*Difco PPLO broth with 1% serum fraction and 1/4000 parts thallium acetate adjusted to pH 7.8 after the addition of erythromycin.

**Values represent the greatest inoculum dilution (expressed as the reciprocal of the log) which initiated growth.

***Mutation rates for these cultures may have been smaller since the concentration of viable units was less than normal (normal is 10^{8} to 10^{9}/ml).

TABLE II

The *in vitro** resistance and mutation rates of PPLO to streptomycin

Prior exposure to streptomycin	Micrograms/ml streptomycin	Mutation rates	Days post inoculation: 3	6	10	13	17	20	24	27	31
			Greatest inoculum dilution which initiated growth								
	0		2**	5	7	7(a)	7	7	7	7	7
	0.1		2	5	7	7(b)	7	7	8	8	8
	1.0		1	3	6	6(c)	6	6	6	6	6
Unexposed	10.0	$1/10^{6}$***	1	1	1(d)	1	1	1	1	2	2
	100.0		1	1	1(e)	1	1	1	1	1	1
	1,000.0			1	1(f)	1	1	1	1	1	1
	10,000.0					1	1	2	2(g)	2	2
	0		2	6	7	7	7	7	7	7	7
	0.1		2	6	7	7	7	7	7	7	7
Unexposed,	1.0		2	4	5	6	7	7	7	7	7
subcultured	10.0	$1/10^{6}$***	1	1	1	1	1	1	1	1	1
from tube (a)	100.0				1	1	1	1	1	1	1
	1,000.0				1	1	1	1	1	1	1
	10,000.0						1	1	1	1	1
	0		4	6	9	9	9	9	9	9	9
	0.1		4	6	8	8	9	9	9	9	9
Exposed,	1.0		3	6	7	8	8	8	8	8	8
subcultured	10.0	$1/10^{8}$	1	1	1	1	1	1	1	1	1
from tube (b)	100.0					1	1	1	1	1	1
	1,000.0					1	1	1	1	1	1
	10,000.0					1	1	1	1	1	1
	0		3	7	9	9	9	9	9	9	9
	0.1		3	7	9	9	9	9	9	9	9
	1.0		3	5	7	8	8	8	8	8	8
Exposed,	10.0	$1/10^{8}$	1	1	1	1	1	1	1	1	1
subcultured	100.0				1	1	1	1	1	1	1
from tube (c)	1,000.0										

Prior exposure to streptomycin	Micrograms/ml streptomycin	Mutation rates	Days post inoculation: 3	6	10	13	17	20	24	27	51
			Greatest inoculum dilution which initiated growth								
Exposed, subcultured from tube (d)	0	****	4	6	6	6	6	6	6	6	6
	0.1		4	5	6	6	6	6	6	6	6
	1.0		3	4	4	6	6	6	6	6	6
	10.0		1	1	3	3	3	3	3	3	3
	100.0		1	1	2	3	3	3	3	3	3
	1,000.0		1	1	2	3	3	3	4	4	4
	10,000.0		1	1	2	3	3	3	3	3	3
Exposed, subcultured from tube (e)	0	****	5	6	8	8	8	8	8	8	8
	0.1		3	5	6	7	7	7	7	7	7
	1.0		5	6	6	7	8	8	8	8	8
	10.0		5	5	6	8	8	8	8	8	8
	100.0		5	5	8	8	8	8	8	8	8
	1,000.0		4	4	4	4	6	6	6	6	6
	10,000.0		4	4	6	6	6	6	6	6	6
Exposed, subcultured from tube (f)	0	****	4	5	8	8	8	8	8	8	8
	0.1		4	5	7	7	7	8	8	8	8
	1.0		4	6	6	7	7	7	7	7	7
	10.0		4	4	6	6	7	7	7	7	7
	100.0		3	4	6	6	7	7	7	7	7
	1,000.0		3	5	5	7	7	7	7	7	7
	10,000.0		4	4	6	7	7	7	7	7	7
Exposed, subcultured from tube (g)	0	$1/10^7$	1	5	6	8	8	8	8	8	8
	1.0		1	3	5	6	6	6	6	6	6
	1.0		1	2	2	2	2	2	2	2	2
	10.0		1	1	2	2	2	2	2	2	2
	100.0		1	1	1	2	2	2	2	2	2
	1,000.0		1	1	1	1	1	1	1	1	1
	10,000.0		1	1	1	1	1	1	1	1	1

*Difco PPLO broth with 1% serum fraction and 1/4000 parts thallium acetate adjusted to pH 7.8 after the addition of streptomycin.

**Values represent the greatest inoculum dilution (expressed as the reciprocal of the log) which initiated growth.

***Mutation rates for these cultures may have been smaller since the concentration of viable units was less than normal (normal is 10^8 to 10^9/ml).

****These cultures have exhibited "one-step" mutation to increased streptomycin resistance, and did not mutate further on this their second exposure to streptomycin.

but mutated when subcultured in all concentrations of antibiotic at the rate of 1 in 10^8 to resistance to as much as 10,000 micrograms/ml. Thus, these cultures behaved as the controls but exhibited a somewhat smaller mutation rate perhaps as indicated previously, because a larger number of viable units was assayed. Cultures previously exposed to 10 (tube d), 100 (tube e) and 1,000 (tube f) micrograms/ml of streptomycin contained populations which were refractory to all concentrations (0.1 through 10,000 micrograms/ml) of the antibiotic. These cultures thus exhibited a "one-step" mutation to increased resistance to streptomycin. The culture which had been exposed to the 10,000 microgram/ml (tube g) exhibited only a slight increase in resistance and mutated at the rate of one in 10^7 to increased streptomycin resistance.

Tetracycline (*table 3*). No mutation to increased tetracycline resistance was noted *under the conditions of this experiment* (see discussion). The growth of PPLO was suppressed and retarded directly in relation to the concentration of the antibiotic.

Chloromycetin (*table 3*). No mutation to increased chloromycetin resistance was detected *under the conditions of this experiment* (see discussion). The growth of PPLO was again suppressed and retarded directly in relation to the concentration of the antibiotic.

It is of interest to note the growth pattern of the various PPLO cultures with respect to the concentration of viable PPLO units throughout the experiment. Most frequently maximum growth (as indicated by viable titer) was established within the first three weeks of incubation. However, in some instances as many as 2 or even 3 weeks were required for maximum growth to occur.

DISCUSSION

In a study of this type it is essential, of course, that the the test organism must never have had any contact with antibiotics and be typical of the group of organisms one wishes to study. Fortunately, as far as can be determined, the W strain fulfilled these requirements.

TABLE III

The *in vitro** resistance and mutation rates of PPLO to tetracycline

Prior exposure to tetracycline	Micrograms/ml tetracycline	Mutation rates	Days post inoculation: 3	6	10	13	17	20	24	27	31
			Greatest inoculum dilution which initiated growth								
Unexposed	0	***	4**	4	4(a)	4	4	4	4	4	4
	0.1		2	2	2(b)	2	2	2	2	2	2
	10.0					3	3	3(c)	3	3	3
	100.0										
Unexposed, subcultured from tube (a)	0	***	6	9	9	9	9	9	9	9	9
	0.1		1	2	2	2	3	3	3	3	3
	10.0		1	1	1	1	1	1	1	1	1
	100.0										
Exposed, subcultured from tube (b)	0	***	4	6	6	6	6	6	6	6	6
	0.1		1	5	6	6	6	6	6	6	6
	10.0				1	1	2	2	2	2	2
	100.0										
Exposed, subcultured from tube (c)	0	***	3	3	3	5	6	6	6	6	6
	0.1		3	3	3	4	4	4	4	4	4
	10.0							1	1	1	1
	100.0										

* Difco PPLO broth with 1% serum fraction and 1/4000 parts thallium acetate adjusted to pH 7.8 after the addition of tetracycline.

** Values represent the greatest inoculum dilution (expressed as the reciprocal of the log) which initiated growth.

*** Growth of these cultures was suppressed in direct relation to antibiotic concentration; thus, under these conditions (single exposure to and 10 fold increases in antibiotic concentration) no mutation to increased resistance was detected.

TABLE IV

The *invitro** resistance and mutation rates of PPLO to chloromycetin

Prior exposure to chloromycetin	Micrograms/ml chloromycetin	Mutation rates	Days Post inoculation 3	6	10	13	17	20	24	27	31
			Greatest inoculum dilution which initiated growth								
Unexposed	0	***	3**	5	5	5(a)	5	5	5	5	5
	0.1		3	4	4	4(b)	4	4	4	4	4
	10.0		2	2	2(c)	2	2	2	2	2	2
	100.0										
Unexposed subcultured from tube (a)	0	***	5	7	8	9	9	9	9	9	9
	0.1		4	7	7	8	8	8	8	8	8
	10.0		2	3	3	4	4	4	4	4	4
	100.0				1	1	1	1	1	1	1
	100.0										
Exposed subcultured from tube (b)	0	***	5	5	7	7	8	8	8	8	8
	0.1		3	3	3	4	4	4	4	4	4
	10.0		1	1	2	3	3	3	3	3	3
	100.0										
Exposed, subcultured from tube (c)	0	***	5	5	5	5	6	6	6	6	6
	0.1		3	3	3	3	5	5	5	5	5
	10.0		2	2	2	2	3	3	3	3	3
	100.0										

* Difco PPLO broth with 1% serum fraction and 1/4000 parts thallium acetate adjusted to pH 7.8 after the addition of chloromycetin.

** Values represent the greatest inoculum dilution (expressed as the reciprocal of the log) which initiated growth.

*** Growth of these cultures was suppressed in direct relation to antibiotic concentration; thus, under these conditions (single exposure to and 10 fold increases in antibiotic concentration) no mutation to increased resistance was detected.

It is apparent that the W strain of PPLO exhibited "one-step"[2] mutation to streptomycin and rapid "step-wise"[1] mutation to erythromycin at mutation rates of approximately 1 in 10.[8] This mutation rate is within the normal range of 1 in 10^8 to 10^{12} found for mutation to streptomycin resistance[9] by various bacteria.

Since studies of this type frequently are used to compare one antibiotic or type of antibiotic against another for therapeutic purposes, it must be emphasized that this study was designed to determine the mutation characteristics of PPLO to increased antibiotic resistance following only a single exposure to the antibiotic. It must also be noted that the test antibiotic concentrations were spaced in 10 fold increments. If PPLO develop antibiotic resistance as bacteria do (the evidence presented in this paper indicates that this is true for streptomycin and erythromycin), mutants to increased tetracycline and chloromycetin resistance would not be detected by the methods used in this experiment, but would be detected only if antibiotic concentrations were increased in two fold increments.[1] Therefore, the only valid comparative inferences that can be made from this study are that streptomycin in particular must be used at high initial levels in order to prevent the selection of resistant PPLO and even then resistant forms may develop; and, that erythromycin must also be used with discretion and only at reasonable therapeutic levels. Although it cannot be concluded from this experiment, it is well to remind ourselves at this point that the *in vivo* use of any antibiotic at subthereapeutic levels over a period of time invites the selection of antibiotic resistant microorganisms.[10]

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SUMMARY

One-tenth, 1.0, 10, 100, 1,000 and 10,000 microgram/ml concentrations of streptomycin, erythromycin, tetracycline and chloromycetin were tested for their ability to inhibit the growth of medium-adapted 72 hr. serially diluted populations of the "W" strain of Mycoplasma. The presence or absence of growth as judged by visible turbidity was recorded twice weekly for one month following inoculation. Tubes containing the smallest population of Mycoplasma capable of initiating growth in each of the antibiotic dilutions were sub-cultured and again observed as described above. The data thus obtained indicated that streptomycin resistance increased in one-step and that 1 in 10^8 cells were resistant to high concentrations of streptomycin; and, erythromycin resistance occurred in a step-wise manner and that 1 in 10^8 cells were resistant to increased concentrations of erythromycin. Mutation to tetracycline or to chloromycetin resistance was not detected, probably because antibiotic concentration was increased in 10 fold increments and the culture was exposed to the test antibiotics only once; however, a gradual suppression of growth in direct relation to antibiotic concentration was observed.

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THE EFFECT OF PARENTAL IMMUNITY AND AGE ON THE RESPONSE TO INFECTIOUS LARYNGOTRACHEITIS VACCINATION*

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ALTHOUGH vaccination against infectious laryngotracheitis (IL) has been practiced for many years, there are still many unknown factors regarding the mechanisms of this process. The quality of vaccine has been greatly improved over the past years by the use of the embryonated chicken egg in its preparation. The immunity produced has always been said to be of the most durable type. It has only been in the last few years that interest has been rekindled in finding better methods of applying the vaccine as well as in determining the most efficient age of birds for vaccination. Does parental immunity exist in this disease and if so, does it have an influence on active immunization? What is the effect of age on the ability of the bird to respond to a vaccine? The research reported here is an attempt to obtain information about some of these factors.

MATERIALS AND METHODS

The part of the report which deals with parental immunity is essentially a continuation of the previously reported work by Benton *et al.*[1] and materials and methods are either similar or identical to those already described. This work was

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divided into two experiments. Experiment I was designed to study the influence of parental immunity on the reaction of young chickens to I.L. vaccination. Chicks were obtained from the same parent flock used by Benton.[1] This adult group of 45 Single Comb White Leghorn chickens was divided into two groups at 30 weeks of age. Group I included 18 females and four males and was not vaccinated for IL. Group II, containing 19 females and four males, was vaccinated with a commercial IL vaccine by the vent brush technique at 30 weeks of age. This vaccine had a 50 percent embryo infective dose (EID_{50}) of greater than $10^{-6.50}$. The immunity of each group was determined from time to time by the serum neutralization (SN) test which was done in the manner described by Benton *et al.*[2] except that the eggs of the SN test were examined on the 7th day after incubation. The serums were stored frozen at –15 C and were not heat inactivated before use in the SN test. Eggs from these two groups of adult birds were saved during the 7th week after vaccination and stored in an egg cooling room until set for incubation and hatching.

The chicks from each group were hatched separately. Each chick was identified by wing banding. Vaccination of the chicks in this experiment was accomplished by the cloacal drop method with a 1:10 dilution of a virus derived from the same commercial vaccine as used on parents. This virus was passed in embryonated chicken eggs and the vaccine was prepared as described below. At the time of embryo mortality or on the 5th day after injection, the eggs were removed from the incubator and chilled. The chorio-allantoic membranes (CAM) of the embryos with typical lesions of IL were removed aseptically. A 1:2 weight-volume dilution was made by grinding the membranes in the harvested chorio-allantoic fluid (CAF) from the same eggs. For the vaccination of chicks, this material was diluted 1:5. Thus the final vaccine was a 1:10 dilution of the original membrane on a weight-volume basis. The titer of each virus used for challenge or vaccination was determined in embryonated eggs. Each vaccinated bird was examined for a cloacal reaction "take" on the 3rd day after vaccination.

The chicks within each progeny group were divided into three lots and vaccinated at 8, 14, and 21 days of age, respectively to determine the influence of age on vaccination. The vent drop method was used with a vaccine of an EID_{50} titer greater than $10^{-4.50}$. Immunity or susceptibility was determined by an intrasinus challenge (right sinus) of 0.1 ml of a 10^{-1} dilution of the same virus used for the vaccine (EID_{50} $10^{-4.80}$ for 8 days, all others $10^{-4.05}$). This challenge was done 28 days after vaccination. Several chicks from each age group picked at random (immune and non-immune parents) were held in isolation as non-vaccinated controls. These control chicks were also challenged at the same time as the vaccinated chicks. Blood samples were taken at 21 days post vaccination to determine the neutralizing index of the serum. Susceptibility to challenge was determined by examining the birds on the 3rd through the 6th day after the intrasinus inoculation. Swelling of the sinus (with or without nasal exudate and/or lacrimation) indicated susceptibility.

Experiment II was designed to test the immunity to IL when it was combined with Newcastle and infectious bronchitis virus and applied by the vent drop technique at 2 weeks of age. In this work, 240 straight run chicks from the University of Delaware Poultry Farm were used. They were divided into three groups as follows:

Group I—100 birds vaccinated by the vent drop method at 2 weeks of age with a vaccine which contained the B_1 type Newcastle virus, the modified Luginbuhl (Mass.) strain of infectious bronchitis virus and laryngotracheitis virus.

Group II—100 birds vaccinated as Group I, except that a B^1 strain Newcastle virus was used instead of the B^1 type Newcastle virus.

Group III—40 birds not vaccinated and used as controls.

The vaccinated birds were examined for laryngotracheitis takes on the 5th day after vaccination. At 5 and 10 weeks of age birds were removed from each of the three groups for challenge as follows: 20 birds for IL challenge, 20 for New-

castle challenge. The IL intrasinus challenge was used as in experiment I. The Newcastle challenge was accomplished by the intranasal instillation of one drop of GB Newcastle virus in a $10^{-3.0}$ dilution.

Another part of the experiment included four lots of birds which were obtained from commercial broiler flocks. These birds had been vaccinated against IL by the vent drop technique at 7 days of age. These four lots represented 34,000 birds. They were transported to the laboratory and were challenged for IL and Newcastle disease at 32 days of age, at 45 days of age and at 66 days of age. Ten birds were included in each test lot.

RESULTS

Eperiment I. Of the chicks vaccinated at 8 days of age, all 10 from the non-immune parents showed a vaccination take while nine of 10 from immune parents exhibited a take. The results of SN tests on serum obtained 19 days after vaccination as well as the results of intrasinus challenge on the 27th day after vaccination are shown in chart 1. The challenge virus had a titer of $10^{-4.8}$.

Of the chicks vaccinated at 14 days of age, six of the 10 from the immune parents exhibited a vaccination take while eight of the 10 from non-immune parents showed takes. The challenge virus had an EID_{50} of $10^{-4.05}$.

Of the chicks vaccinated at 21 days of age, all birds of both groups showed takes following vaccination.

It is apparent that only minor differences existed in the ability of these chicks to react to the vaccination stimulus. The sinus reaction of a bird could not be related directly to the neutralization index of the serum.

Experiment II. Nine of 20 chicks in Group I and six of 20 in Group II of chicks vaccinated at 2 weeks of age by the vent drop technique were susceptible to IL at 5 weeks of age and almost all were susceptible at 10 weeks of age. The birds resisted a Newcastle virus challenge at both ages (table 2).

The result of SN tests for IL on these same birds is shown in table 3. It is to be noted that results of the SN tests did

TABLE I

Results of challenge and serum neutralization tests of chicks from immune and non-immune parents

Source of chicks	Vaccination age	Results of intrasinus challenge Number susceptible over number challenged	Serum neutralizing index	Sinus reaction same chick
			0.27	N
Non-Immune			1.00	N
parents	8 days	1/10	0.00	N
			0.33	N
Immune			0.00	N
parents	8 days	3/10	1.00	+
			0.00	+
Not			1.00	+
vaccinated	—	5/5	1.00	+
Non-			1.67	N
immune			1.54	N
parents	14 days	1/9	1.34	N
			1.17	N
Immune			1.34	N
parents	14 days	1/10	1.67	+
			1.43	+
			0.67	+
Not			0.92	+
vaccinated	—	5/5	0.67	+
Non-immune			0.00	N
parents	21 days	1/10	1.00	N
			1.35	N
Immune			1.00	N
parents	21 days	0/10	0.84	N
			1.00	+
			0.17	+
Not			0.33	+
vaccinated	—	6/6	0.67	+

TABLE II

Challenge results of chicks vaccinated by vent drop method at 2 weeks of age

	Infectious laryngotracheitis challenge*		Newcastle challenge*	
	Age 5 wks.	Age 10 wks.	Age 5 wks.	Age 10 wks.
Group I	9/20	19/20	0/20	0/20
Group II	6/20	17/20	2/20	1/20
Group III	5/5	4/4	5/5	4/5

* Number Susceptible/Number Challenged.

not show any marked degree of immune response and are similar to the results of the challenge.

The results of the challenge of birds vaccinated as commercial broiler flocks are shown in table 4. Again, the chicks vaccinated by the vent drop method at 7 days of age developed neither a very durable nor strong immunity.

TABLE III

Serum neutralization test of birds vaccinated with infectious laryngotracheitis at 2 weeks of age

	Neutralization indices		
	Pre vaccination 2 wks. of age	5 wks. of age	10 wks. of age
Group I			
Bird 1	0.00	0.00	1.13
2	0.17	1.13	1.25
3	0.49	0.00	—
4	0.42	—	—
5	0.67	—	—
Group II			
Bird 1	—	0.13	1.13
2	—	0.80	1.00
3	—	0.00	1.63
4	—	—	—
5	—	—	—
Group III			
Bird I	—	0.00	0.00
2	—	0.00	0.95
3	—	—	0.54

TABLE IV

Challenge of commercial broilers which were vaccinated at 7 days of age with IL

	Infectious laryngotracheitis challenge*			Newcastle challenge*		
LOT	32 days	45 days	66 days	32 days	45 days	66 days
A	0/5	2/5	4/5	0/5	0/5	0/5
B	0/5	4/5	5/5	0/5	0/5	0/5
C	—	—	4/5	—	—	0/5
D	—	—	5/5	—	—	0/5
Control	5/5	5/5	4/5	5/5	5/5	4/5

* Number Susceptible/Number Challenged

DISCUSSION

Although the number of chicks involved in this study of parental immunity to IL is small, there appears to be no difference in the response of chicks to a vaccine regardless of the immune state of the parents. Perhaps if a higher degree of

parental immunity had been present, an effect on the reaction to vaccination could have been noted.

It is the opinion of the writers that birds 2 weeks of age or younger do not respond as fully to a vaccination stimulus as do chicks 4 weeks of age or older. This fact must be kept in mind when IL vaccination is to be done. In emergencies, in areas where IL is an active problem, and early (2 weeks or less) vaccination is done, revaccination at a later date (6 to 7 weeks of age) is recommended.

CONCLUSIONS

The differences in the ability of chicks from IL immune and non-immune parents to react to IL vaccination were determined. There were only minor differences in the ability of the chicks from these two parental sources to react to IL vaccination. In this experiment it was not possible to correlate the sinus reaction to IL virus with the serum neutralization index of the bird.

The ability of chicks at one week and at two weeks of age to react to the vent drop method of IL vaccination was tested. Chicks vaccinated at this age did not develop a strong, durable immunity.

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A QUICK METHOD FOR THE DIAGNOSIS OF AVIAN POX AND INFECTIOUS LARYNGOTRACHEITIS

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DURING 1959, there were significant numbers of outbreaks of avian pox and infectious laryngotracheitis in Massachusetts. Workers at this laboratory have been particularly concerned about these diseases not only because of the numbers of diagnoses but also of the possibility of not recognizing the atypical, mild forms of laryngotracheitis and the more unusual cases of pox where lesions are found sometimes only in the trachea. Early and accurate recognition of both these diseases is necessary if an effective control program is to be instituted. Chicken embryo inoculations for virus isolations take a minimum time of 3 to 6 days and recent tissue culture techniques employed at this laboratory have not lessened the time.[3] Histopathologic examination of affected tissues processed by classical methods taking a minimum of 24 to 48 hours have yielded a diagnosis in nearly all cases where several birds have been submitted. Therefore much emphasis has been placed on a more rapid, yet simple and satisfactory method of processing affected tissues. Several reports in the literature cite rapid methods.[1,2,4] In our hands we prefer and use the method outlined below because it is simple, rapid and accurate.

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MATERIALS AND METHODS

1. Place small pieces of affected trachea or skin in the following solution at 56 C (paraffin oven) for 30 minutes.

95% alcohol saturated with picric acid	75 cc.
Formalin 40%	15 cc.
Glacial acetic acid	5 cc.

2. Dehydrate in two changes of absolute alcohol (56 C) for a total of 20 minutes.
3. Clear in two changes of toluene (56 C) for a total of 30 minutes.
4. Place tissues in melted Tissuemat (Fisher) (58 C) for 20 minutes and then imbed.
5. Cut sections 6 microns thick and place them on slides which have been smeared with Mayer's albumen. Dry with mild heat for 20 minutes.
6. Bring sections through xylol and alcohol, stain with hematoxylin and eosin, dehydrate, clear, mount and label.

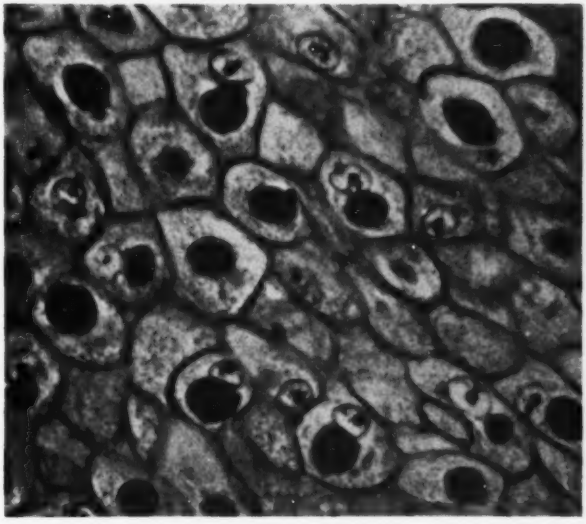

Fig. 1. Photomicrograph of a section of skin with intracytoplasmic inclusions of avian pox stained with hematoxylin and eosin (430×).

RESULTS AND DISCUSSION

Intracytoplasmic inclusions for avian pox (figure 1) and intranuclear inclusions for laryngotracheitis (figure 2) have been demonstrated with accuracy when the above method was compared to the longer, classical methods with Bouin's or Zenker's fixatives. The satisfactory demonstration of cellular detail and the preservation of histological architecture with this technique were advantages when compared to the smear

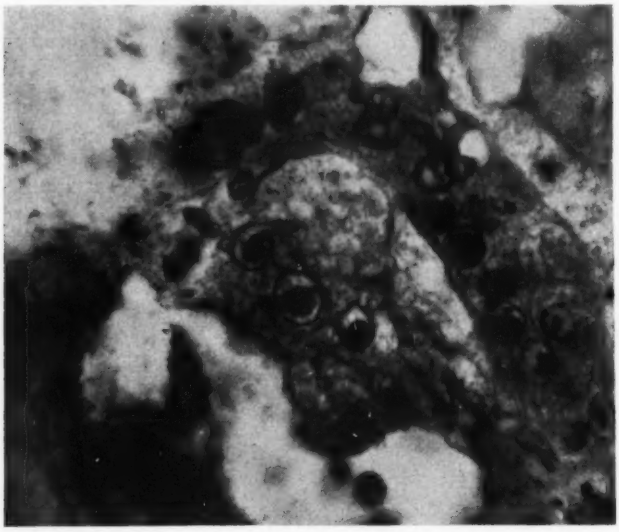

Fig. 2. Photomicrograph of a section of trachea with intranuclear inclusions of infectious laryngotracheitis stained with hematoxylin and eosin (900×).

methods which have been described.[1,2] There was, however, some shrinking of tissue. Intranuclear inclusions for laryngotracheitis were demonstrated as early as the second day after intratracheal inoculation and till such time as the tracheal mucosa sloughed (approximately 4 days). Cytoplasmic inclusions for avian pox were demonstrated as early as 4 days after inoculation, but the duration of these lesions varied considerably.

SUMMARY

A rapid, histologic method involving the use of a solution which fixes and dehydrates tissues simultaneously has been described. With the use of hematoxylin and eosin stains, intranuclear inclusions for laryngotracheitis and cytoplasmic inclusions for avian pox were demonstrated readily in less than 3 hours time.

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FURTHER STUDIES OF VIRUS HEPATITIS OF TURKEYS*

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A virus-like agent was first reported in 1959[1,2] to be the cause of a hepatitis in turkeys. The reports were in close agreement concerning the characteristics of the causal agent and the pathological process in poults. It appeared that simi-

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lar, if not identical, agents were involved in the isolation reported from Canada and the four isolations reported by us.

Additional field cases have been studied to gain information concerning the incidence and economic importance of the infection. Experimental trials were conducted to improve diagnostic methods and to gain knowledge of the epizootiology of the disease. A total of 17 field infections of the turkey hepatitis virus (THV) has been diagnosed in Massachusetts laboratories since the initial report. Eleven of the cases occurred in poults less than 5 weeks of age; mortality varied from 1 to 25 percent. Other disorders, including concomitant infections, were found in several instances. Two cases occurred between 6 and 8 weeks of age but were considered to be an incidental finding.

Four diagnoses were made in mature stock at the time of slaughter.* All groups of birds, ranging in number from 590 to 1400, were considered to be in excellent condition antemortem. At postmortem inspection, liver condemnations varied from an estimated 30 to 90 percent. A majority of the condemned livers exhibited numerous small, gray lesions visible on the surface and scattered throughout the parenchyma. Large granulomatous lesions were also observed in a few livers.

MATERIALS AND METHODS

Turkeys and eggs were secured from flocks maintained under commercial conditions. Poults were held in wire floored batteries.

Either undiluted yolk-propagated virus or preparations from affected turkey livers were used for inoculums. The latter was prepared by grinding affected livers in four parts nutrient broth in a Waring blendor, centrifuging to remove large particulate matter, and filtering through a Seitz EK filter. Methods for preparing tissues for isolation, for embryo propagation of the agent, and for holding harvested materials have been described.[2]

*The assistance of Dr. Alexander Don of the Inspection Branch, Poultry Division, Agricultural Marketing Service, USDA, in collecting specimens and furnishing histories is gratefully acknowledged.

Serum neutralization (SN) tests were performed according to the method of Cunningham.[3] Both yolk and a filtered liver preparation were used as a virus source; these materials demonstrated embryo infective dose$_{50}$ (EID_{50}) titers of $10^{-2.6}$ and $10^{-3.1}$ respectively. Antigen for the complement fixation (CF) test was prepared from isolate #463 according to method 3 of Topping and Shepard,[4] except that yolk contents from the pool previously described were used rather than yolk sacs. CF tests were conducted as described in the Manual of Microbiological Methods.[5]

Isolation attempts were considered positive if typical lesions and mortality occurred in embryos injected with material from intentionally exposed birds. Field isolates were also checked for turkey infectivity before being considered positive. Three blind passages in embryos were made with liver preparations from the field cases in mature birds.

In some trials, gross lesions were scored on a 0 to 4 basis depending upon the presence and severity of hepatic involvement visible on the surface. The presence or absence of pancreatic lesions was noted.

EXPERIMENTS AND RESULTS

Most isolation attempts have been successful from livers exhibiting extensive lesions from field cases in flocks less than 6 weeks of age. Characteristic embryonic mortality and lesions have usually been secured on the first or second passage. No isolations were secured from the field cases in mature birds, although characteristic lesions were prominent.

Fifteen 11-week-old turkeys were injected intravenously with 0.2 ml of yolk harvest from the second embryo passage of isolate #356. At 48 hour intervals, pairs of birds were sacrificed. Lesions were noted, and pools of tissues saved for isolation. Feces were collected by expressing from the cloaca. The results are summarized in table 1.

The gross liver lesions noted in one bird 2 days post infection (PI) represent the earliest lesions following exposure. Although the agent was occasionally isolated from bile, blood, spleen and kidney, the liver appeared to be the organ of choice

for isolation purposes, and occasionally yielded the virus although no gross lesions were detected. Feces were also a rather dependable source of the virus.

Ten, 8-month-old commercial breeding turkey hens were secured to determine susceptibility to the virus and clinical effect. They were injected intravenously with 0.5 ml of the fifth embryo passage of isolate #356. Randomly selected birds were sacrificed at 48 hour intervals between the 5th and 21st day post-injection. Lesions were noted and sections of liver saved for isolation. Results are summarized in table 2.

TABLE I

Presence of turkey hepatitis virus (THV) in several locations at intervals following infection

		Virus isolation					
Days P.I.	Liver lesions	Liver	Bile	Feces	Blood	Spleen	Kidney
2	1/2*	+	—	—	—	0	0
4	2/2	+	—	+	+	+	+
6	2/2	+	—	—	+	0	0
8	2/2	+	—	+	—	0	0
10	2/2	+	—	+	—	0	0
12	0/2	+	+	+	+	—	+
14	0/2	+	—	+	—	0	0
16	1/1	+	+	+	—	0	0

0 = not done.
** numerator = number of birds with lesions
denominator = number of birds examined.

TABLE II

Response of turkey hens to intravenous infection of turkey hepatitis virus (THV)

Days P.I.	Liver lesions	Ovarian condition	Isolation from liver
5	2/2**	Active	—
7	1/1	Inactive	—*
9	1/1	Active	+
11	1/1	Active	—
13	1/1	Inactive	—
15	1/1	Inactive	+
17	0/1	Active	—
19	1/1	Active	—
21	1/1	Inactive	+

* Isolation from contents of retrogressing follicle.
** numerator = number of birds with lesions
denominator = number of birds examined.

The birds remained clinically normal. Egg production was poor because of age and season but did not appear to decrease following exposure. Isolation from retrogressing ovarian follicular contents was accomplished in one instance. The agent was not consistently isolated from grossly affected livers from this group of mature hens.

Several trials were conducted to ascertain the development of immunity following recovery from artificial infection in poults. A representative trial is detailed. Seventy-two poults were divided into two groups; one was held in isolation and at 5 days of age the other was injected intraperitoneally (IP) with 0.2 ml of the third embryo passage of isolate #463. Sixteen of the injected birds were killed on the 7th day PI. All of the specimens exhibited extensive liver lesions and 14 also had grossly demonstrable pancreatic lesions. A pool of these livers was used to prepare a filtrate for challenge following recovery. This preparation had an EID_{50} titre of $10^{-3.1}$; the initial yolk inoculum had a titre of $10^{-2.6}$. At 21 days PI, which previous experience had indicated was usually adequate to allow recovery from gross lesions, both groups were challenged by intravenous injection of 0.2 ml of liver filtrate and were sacrificed and examined 7 days later. Liver lesions were scored and the presence or absence of pancreatic lesions was noted. Results are summarized in table 3.

TABLE III

Response of recovered turkeys to intravenous re-exposure to turkey hepatitis virus (THV)

	Liver lesions	Lesion score		Pancreatic lesions
		Group total	Bird average	
Re-exposed	11/22*	11	0.5	1/22
Control	31/34	55	1.6	18/34

* numerator = number of birds with lesions
denominator = number of birds examined.

Although liver lesions were found in one-half of the re-exposed group, visible changes consisted of no more than one or two small foci. Slight pancreatic lesions were found in only one bird. Liver lesions were present in all but three of the 34 in the susceptible group and were frequently very extensive.

Distinct pancreatic lesions were present in over half of this group. A similar pattern of limited reaction following re-exposure was demonstrated in birds held as long as 37 days between initial exposure and challenge. In the latter group occasional single or multiple small hepatic granulomas were found at necropsy. In one group no lesions were found on challenge following recovery.

One trial conducted to ascertain possible transmission of THV as a consequence of eggshell contamination. A group of 100 unincubated turkey eggs were warmed by incubating for 2 hours at 37 C. They were then immersed for 30 minutes in an aqueous suspension at 5 C containing 2000 ppm chlortetracycline and 1 percent liver virus preparation of isolate #463 with an EID_{50} titre of $10^{-3.0}$. Forty unincubated eggs from the same source were injected with 0.2 ml of the same virus preparation. Infertile eggs from both sources were discarded at the 7th day of incubation, and fertile eggs were candled daily thereafter. Embryos from 12 of the 17 fertile injected eggs succumbed and exhibited characteristic lesions. The remaining five embryos appeared normal when examined on the 27th day.

Sixty-eight fertile eggs were found in the dipped group at the 7th day of incubation. Eleven embryos died during later incubation and eight embryos pipped and failed to hatch. Yolk or liver harvest from each dead or pipped embryo was injected into the yolk sac of six chicken embryos; no virus isolations were made.

The 49 poults were held in isolation and groups of randomly selected poults necropsied at weekly intervals; no lesions of the viral infection were found. At the end of the 4th week, 10 remaining poults and an equal number of poults of the same age reared in isolation were challenged by intravenous injection of 0.1 ml of yolk-propagated isolate #463. The birds were necropsied on the 7th day PI. The severity and extent of lesions were essentially identical in the two groups and were as extensive as usually produced in susceptible stock of this age.

Serological reactions. A group of ten 7-day-old poults was injected IV with 0.2 ml of yolk material from isolate #463. Seven days later characteristic lesions were observed in five birds which were necropsied. A second IV injection of the remaining birds was made 35 days later and blood was collected for testing 7 days after the last injection. Susceptible serum was secured from unexposed birds of the same age. Neither serum neutralizing nor complement fixing activity was detected in any of these serums.

DISCUSSION

Isolation from livers has been accomplished with relative ease in most instances of field infections of poults. Usually a high proportion of the embryos die on the first passage. Similar ease of isolation has been the rule from livers of experimentally infected poults. Mature, susceptible stock developed lesions following exposure, but isolation attempts frequently were unsuccessful. The latter results suggest that in the absence of virus isolation, pathological lesions must be relied upon for a diagnosis in mature stock. On this basis, failure of isolation from the four field cases in asymptomatic mature birds is not surprising. The diagnoses appear valid inasmuch as typical gross and microscopic lesions were found, and the behavior of the infection was compatible with that anticipated.

Initial reports indicated that the lesions occurring in field infections were more severe and extensive than those produced following experimental infection. Mortality was also observed in field cases, but neither morbidity nor mortality had been reported following experimental infection.[1,2] Several of the field infections reported in this communication failed to produce clinical signs and exhibited liver lesions of no greater severity than those produced experimentally. It should be added that a considerable number of field and experimentally infected poults have been observed with degenerative, hepatic lesions resembling those produced by *Histomonas meleagridis*. Gross pancreatic lesions were an inconstant finding, and usually occurred only in specimens with extensive hepatic involvement.

Laboratory and field observations to date with TVH indicate that the disease is highly contagious. Examinations of histories from field cases strongly suggests that in most instances the disease is subclinical. In clinical cases, concomitant disorders are frequently found. Only occasionally are losses severe in the absence of other recognized disorders. In the latter case the role of possible parental immunity needs clarification.

Failure to demonstrate either complement fixation or serum neutralizing activity should be regarded only as lack of reaction under the specific circumstances. A measurable immunity, as demonstrated by response to challenge, exists for at least a short period following infection. Further investigation is necessary to determine the level and duration of such immunity.

A small group of turkey hens was infected, and many remained in active egg production throughout the observation period. It was demonstrated that a viremia exists during a considerable period of the infection. These results together with the isolation from the contents of an ovarian follicle, albeit retrogressive, suggest the possibility of egg transmission of the agent as one means of dissemination. Contamination of egg shells with THV prior to incubation failed to infect embryos or poults developing from such eggs.

It is not known if the hepatic granulomas found in field cases, as well as in one experimental group, are related to the virus under investigation.

SUMMARY

A number of additional field infections of turkey hepatitis virus have been diagnosed. These cases represented a variety of ages including mature stock, and in some instances were subclinical. In the latter cases lesions were relatively slight and were comparable to those produced experimentally. Clinical cases have not been observed in birds older than 5 weeks. Contamination of the shells of turkey eggs with THV prior to incubation failed to produce infection in embryos or poults subsequently produced.

A considerable degree of immunity develops following infection, but the duration is undetermined. Preliminary trials with the CF and SN test have been unsuccessful as a measure of immunity.

Egg transmission is suggested as one means of dissemination of the virus.

The apparent common occurrence of the infection in Massachusetts suggests that a similar situation may occur in other areas.

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A SENSITIVE WHOLE BLOOD TEST FOR *MYCOPLASMA GALLISEPTICUM**

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PRESENT methods of serological testing for *Mycoplasma gallisepticum* requires the submission of blood samples to

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the laboratory for examination. This process is lengthy, time consuming and non-specific false positive results are frequent after storage of the serum samples.

A whole blood (WB) test devised by Jacobs *et al.*[1] provided a rapid method of detecting antibodies against the agent. When this antigen was used, however, the results were inadequate because the agglutinated particles were small and not easily distinguishable from the blood-antigen mixture. Hence, modification of the antigen was necessary in order to obtain early desirable reactions.

MATERIALS AND METHODS

The antigen was grown by the method described by Jungherr *et al.*[2] The concentrated antigen was then standardized with a PhotoVolt Lumetron Colorimeter, model 410A, with a 420 mμ filter to a 78 percent light transmission reading, corresponding to a McFarland density of 1, concentrated 24 times and resuspended in a 0.175 M solution of sodium chloride. The pH of the antigen was adjusted to 6.2-6.4 with 0.1N NaOH and then stained by the addition of 0.2 ml (per hundred) of a 6 percent alcoholic crystal violet solution. The antigen was sonerated in a Raytheon 10 KC sonic oscillator for two minutes at 1.25 amperes.

For serum plate (SP) or WB tests conducted in the laboratory, the following method was used: 0.02 ml of serum or citrated blood was placed on a plate glass mirror, marked off in one inch squares with a 1.0 ml pipette, 0.1 ml of antigen was placed adjacent to the sample and the constituents were mixed thoroughly with an applicator stick. The plate was rotated gently for 3 minutes and the results were read.

For the field WB test, two 4 mm loopfuls or one drop of blood from a syringe was placed on the glass plate. One tenth ml of antigen was added directly to the sample and mixed thoroughly with a loop or applicator stick. The plate was rotated gently and results were read within three minutes.

A positive test is characterized by a purple, granular precipitation usually starting at the periphery of the mixture (figure 1). A negative test shows no change from the homo-

geneous red mixture when whole blood is used or the homogeneous, violet color when serum is utilized.

Temperature has a marked effect on the field WB test. As the temperature decreases, the rate of reaction decreases as shown by an increase in the time required for agglutination and the small size of the agglutinated particles. The optimum temperature for the test is between 70 to 85 F. An artificial source of heat must be supplied when the temperature is below 50 F.

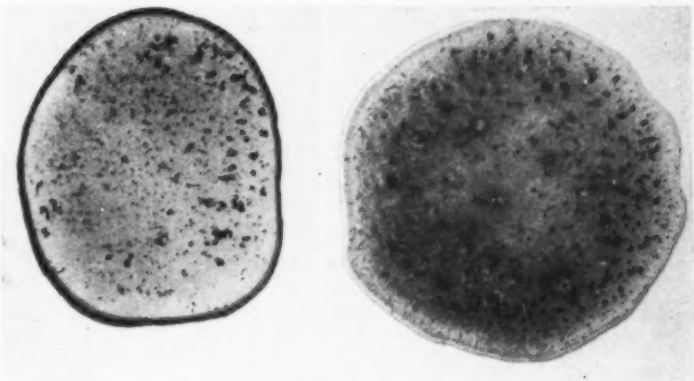

Fig. 1. Left Agglutination with positive serum. Right. Agglutination with positive whole blood.

RESULTS

The results of WB tests conducted by Dr. Norman O. Olson of West Virginia University are recorded in tables 1, 2, and 3. The data in tables 1 and 2 are results of an experiment in which birds were inoculated with various dilutions of *Mycoplasma gallisepticum* by different routes of injection.

There were ten birds per battery, eight inoculated and two contact controls. The birds were tested at 2 weeks of age and found negative. At 2 weeks of age the birds were inoculated by the various routes and dilutions listed. The inoculum was 0.25 ml of broth culture of *Mycoplasma* for all routes except the vent which was one drop. The birds were challenged intrasinusordally with 0.24 ml of a broth culture of *Myco-*

TABLE I

Percent reactors with whole blood antigen and percent isolation by culture at six weeks of age

	Percent positive - percent isolation Dilution of inoculum						Controls*		
	0	1	2	3	4	5	Same room	Same battery	isolated
I.V.	100/70	100/38	88/43[1]	100/14	100[2]/0	100/25	38/0	17/0	—
I.M.	100/25	88/12	88/12	25/0	25/0	50/38	25/0	8/0	—
Pad	100/25	100/0	100/25	100/0	100/25	88/0	25/0	41/0	—
Vent	0/12	12[2]/12[1]	38/0	12/0	38/0	25/0	12/0	8/0	—
I.P.	100/0	100/12	88/25[1]	100/38	75/38	86/40	25/0	41/17	—
Sinus	100/50	100/62	100/50	100/38	100/75	100/12	—	75/75[4]	%

(Courtesy of N. Olson, West Virginia)

[1] - One bird negative on plate but a positive isolation from trachea.
[2] - One showed a weak reaction.
[3] - Totals combined for all dilutions.
[4] - Three negative blood tests. PPLO from trachea.
* - None of the controls were inoculated.

TABLE II

Percent reactors with whole blood antigen and percent isolation by culture at eight weeks of age

	Percent positive - percent isolation Dilution of inoculum						Controls*		
	0	1	2	3	4	5	Same room	Same[4] battery	isolated
I.V.	100/0	100/0	100/0	100/0	100/0	100/0	80/70[1]	100/60	—
I.M.	100/0	100/0	100/14	100/12	100/17	100/14	30/42[2]	92/42	—
Pad	100/0	100/0	100/0	100/30[3]	100/17	100/0[3]	30/10[2]	100/80	—
Vent	100/40	100/14	100/0	100/12	100/28	100/12	30/40[1]	100/42	—
I.P.	100/0	100/0	100/0	100/12	100/12	100/0	30/20[2]	75/40[2]	—
Sinus	100/20	100/0	100/0	100/0	100/0	100/0	—	100/30	%

(Courtesy of N. Olson, West Virginia)

[1] - Two negative blood - positive on isolation.
[2] - One negative blood test - Positive PPLO on isolation.
[3] - 50% or more of cultures contaminated.
[4] - Totals combined for all dilutions
* - None of controls were inoculated.

plasma at 6 weeks and vaccinated for bronchitis and Newcastle disease.

The following numbers of organisms per ml for the various dilutions are listed below:

0	216,000,000	10^3	182,000
10^1	23,100,000	10^4	4,001
10^2	1,030,000	10^5	2,190

The challenge dose contained approximately 75,000,000 organisms per ml.

The birds in table 3 were exposed at one day old with one drop of a 72 hour broth culture of *Mycoplasma* and treated with two different levels of chlortetracycline for various lengths of time.

TABLE III

Results of isolation of Mycoplasma from tracheal swabs and reaction with whole blood test for Mycoplasma agglutinins

Duration of treatment in weeks	2	4	6	8	10	10		
	Percent isolation[1]					Percent reactors[2]		
						Positive	Suspicious	Negative
1,000 grams Chlortetracycline per ton of feed								
None*	0	0	0	46	44	40	25	35
None**	81	73	75	19	8	80	15	5
1-10	3	0	15	19	3	5	30	65
3-10	94	27	26	37	8	30	25	45
5-10	86	87	25	10	13	60	15	25
7-10	80	92	92	0	0	35	40	25
3-4	78	29	95	30	47	80	20	0
5-6	90	95	26	43	13	90	10	0
7-8	86	94	97	16	28	75	25	0
1,500 grams Chlortetracycline per ton of feed								
1-10	2.5	0	13	11	6	5	15	80
3-10	90	14	13	8	0	15	25	60
5-10	77	90	24	3	0	15	40	45
7-10	89	90	60	2	3	80	10	10
3-4	77	18	78	47	6	75	20	5
5-6	81	95	26	19	13	90	5	5
7-8	89	98	95	8	3	75	20	5

* Not inoculated with Mycoplasma
** Inoculated with Mycoplasma

(Courtesy N. Olson, West Virginia)

1-Based on 20 birds
2-Based on 10 birds

Comparative serum plate (SP) tests were conducted with the WB antigen and standard plate antigens by Dr. Julius Fabricant of the New York State Veterinary College. The results are recorded in table 4.

Results at the University of Connecticut of WB tests conducted in the field versus isolation (tracheal swab technique) are recorded in table 5.

TABLE IV

Comparison of serum plate results with WB and standard plate antigens

Bird #	WB Pre	A Pre	B Pre	C Pre	WB 1 wk	A 1 wk	B 1 wk	C 1 wk	WB 2 wk	A 2 wk	B 2 wk	C 2 wk	WB 3 wk	A 3 wk	B 3 wk	C 3 wk	WB 4 wk	A 4 wk	B 4 wk	C 4 wk
W24	—	—	±		4†	3	±	1	4	×	—		4	3	3		4	3	±	
W28	—	—	±		4	4	±	2	4	3	3		4	×	±		4	4	1	
W30	—	—	—		4	4	±	3	4	4	±		3	×	±		4	4	1	
W36	—	—	—		1	±	±	±	4	4	3		4	4	2		4	×	2	
W54	—	—	—		3	1	±	1	4	2	4		4	3	3		4	4	2	
W55	—	—	±		—	—	±		4	4	±		4	4	3		4	4	2	
W66	—	—	—		4	×	±		4	4	1		4	×	3		4	×	2	
W73	—	—	—		4	4	±		4	4	1		4	×	3		4	×	2	
W78	—	—	±		4	4	±		4	4	1		4	1	3		4	4	2	
W83	—	—	±		±	—	±		4	4	3		4	×	4		4	4	2	
W96	—	—	±	—	2	2	±	1	4	2	1	1	4	4	2	3	4	4	2	4
W100	—	×	±	×	±	±	±	±	4	3	1	2	4	4	3	3	4	4	1	4
WR74	—	—	—	—	±	1	±	±	4	2	2	2	4	3	3	2	4	4	1	4
WR83	—	—	—	—	2	3	±	3	4	4	1	3	4	3	3	3	4	4	2	4
WR96	—	—	—	—	±	±	—	2	4	3	1	3	4	×	3	×	4	4	2	4

(Courtesy J. Fabricant, Cornell University)

* Birds inoculated into air sac.
WB - Conn. WB Antigen.
A, B, and C - Standard plate antigens.
† ± = suspicious, 1 = weak reaction, 4 = strongest reaction.

TABLE V

Whole blood (WB) serology compared with isolation from trachea

	No. of birds	WB	%	Isol	%
Known neg. flocks	236	0/236	0.0	0/236	0.0
Known pos. flocks	100	89/100	89.0	82/94	87.2

Numerator = number of birds reacting
Denominator = number of birds tested

SUMMARY

1. A WB antigen for the rapid detection of *Mycoplasma gallisepticum* agglutinins is described.
2. The antigen works equally well with blood or serum and can be used in conjunction with the pullorum testing program.
3. The antigen is concentrated to 24 times MacFarland den-

sity 1, suspended in 0.175 M sodium chloride pH 6.2-6.4 and stained by the addition of 0.2 percent of a 6 percent alcoholic crystal violet solution.

4. Comparative tests with the standard plate antigen and with culture isolation show agreement.
5. The WB antigen is most affective at temperatures above 70 F and was developed for use with chickens.

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STUDIES ON PARENTAL IMMUNITY TO INFECTIOUS LARYNGOTRACHEITIS OF CHICKENS

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THE role of parental immunity in several poultry diseases of viral etiology has been well established.[3,6,7] Such passive immunity has been shown to be associated with the presence

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of antibody in the yolk and eventually in the embryo and in the hatched chick. Homologous viral activity has been shown to be reduced when inoculated into embryonated eggs from parent stock which had been rendered immune by vaccination[3] or by the natural disease.[9]

The presence of parental immunity in infectious laryngotracheitis (IL) has not been previously established. The age incidence of the disease and the common method of vaccination being of such nature as to preclude early vaccination except in emergeny situations are perhaps the principal reasons why such work has not been done.

The recent advent of a vent drop method of applying IL vaccine without the nesessity of abrading the mucous membrane[5] and the use of a mild strain of the virus by the respiratory route[1] provides a means whereby birds may be vaccinated at a younger age. Therefore, it appeared desirable to invesigate the existance of parental immunity. The purpose of the experiments reported in this paper was to determine if passive immunity exists in the progeny of birds vaccinated against IL by the use of comparative studies on chicks and eggs from a vaccinated and non-vaccinated group of chickens.

MATERIALS AND METHODS

The chicks and eggs used in these experiments were obtained from two groups of Single Comb White Leghorn chickens, differing only in the fact that one group was vaccinated against the disease while the other remained unvaccinated. The parent stock was reared on the University of Delaware Poultry Farm which has not had an outbreak of IL for many years and which does not follow a vaccination program against the disease. These birds, however, had been vaccinated against Newcastle disease. When the experimental birds were 30 weeks of age, 45 birds were moved to the disease experimental area where they were banded and randomly divided into two groups and assigned to separate colony houses. The first group consisting of 18 females and 4 males remained unvaccinated while the second group consisting of 19 females and 4 males was vaccinated at this time. Vaccination was accomplished by the vent brush method with a commercial vac-

cine with an embryo infective dose$_{50}$ (EID_{50}) titer of $> 10^{-6.50}$. A positive reaction to vaccination was seen in all vaccinated birds when examined 5 days later. Serum for serum neutralization (SN) tests were obtained prior to vaccination from five birds (four females and one male) of each group and at 4, 12 and 20 weeks after vaccination from the same birds. Such neutralization indices were used as criteria of the immune status of each group and are shown in table 1. The results obtained indicate a low level of SN antibodies in the unvaccinated parents and a relatively high level in vaccinated parents.

TABLE I

Neutralization indices (log 10) of serum from vaccinated and non-vaccinated parent stock

Vaccination status	Bird #	Time of serum collection: Pre-vaccination	Post-vaccination: 4 weeks	12 weeks	20 weeks
Non-vaccinated	48	—	0.75	0.68	1.00
	52	0.79	0.78	1.05	—
	54	0.00	0.55	0.30	0.87
	49	0.18	0.78	1.05	0.33
	55	—	0.78	0.68	1.50
Vaccinated	2483	0.18	>1.75	2.18	3.00
	2563	0.00	0.78	1.68	1.67
	2552	0.33	>1.55	>1.85	2.67
	73	0.00	>1.88	>2.18	3.48
	60	0.49	>1.88	>1.18	3.12

Eggs from each group were collected once or twice daily and stored in an egg cooling room until set in an incubator. Group identity was maintained throughout the experiments. Embryonated eggs for virus titration were used after 10 or 11 days of incubation. Both eggs and chicks were used in groups from eggs collected over a specified period of time as will be indicated for each experiment.

The virus used in all aspects of this experiment was derived from the vaccine used on parent stock and consisted of the chorio-allantoic membranes (CAM) suspended in the chorio-allantoic fluids (CAF) harvested from eggs previously inoculated with the virus. With the exception of the use of CAF as a diluent, the method of preparation has been described previously.[1] All virus suspensions were placed in glass ampules, flame sealed, and stored at –15 C until used.

Titration of virus suspensions and SN tests were performed in accordance with a previous report[1] except that embryos were examined 7 days post inoculation rather than 8. The chorio-allantoic sac (CAS) route of inoculation was used and five eggs were used for each dilution. In those instances where diluted serum and yolk were used, the results were corrected for the dilution factor.

Embryo infective titer in eggs. In order to ascertain the influence upon viral activity in eggs from immune parents, several comparative titrations were made. These were accomplished by titrating a virus suspension in eggs from each group simultaneously. Eggs collected during five post vaccination periods were used in a total of five such titrations. The first titration was in eggs collected 4 through 5½ weeks after vaccination, the second, 9 to 11 weeks, the third, 12 to 14 weeks, the fourth, 14 to 16 weeks and the fifth, 23 to 25 weeks.

Sinus infective titer in chicks. In view of the fact that IL virus can be effectively titered in the sinus of chickens,[2] two comparative titrations were made in 8-day-old chicks from each parental source. This was accomplished by inoculating the right sinus with 0.05 ml of selected dilutions of the virus suspension. Five chicks were used for each dilution. The sinus reaction was observed on the 3rd through the 6th day and recorded. From the resulting data, a sinus infective $dose_{50}$ (SID_{50}) was calculated employing the method of Reed and Muench.[8] The first titration employed chicks from eggs collected 4 to 7 weeks after vaccination while the second used chickens collected 7½ to 9½ weeks after vaccination.

Yolk neutralization. Neutralization studies were performed with yolk from eggs collected from each group. The yolk was collected on the same day the eggs were collected or on the following day after remaining overnight in a refrigerator. All yolk was diluted 1:5 with sterile saline at the time of collection, vigorously mixed and then stored at –15 C until used in the test. Yolk was obtained on the 7th, 14th, 21st, 28th, 56th, 84th and 96th day after vaccination from five randomly selected eggs obtained from each group.

Progeny serum neutralization. Serum neutralization

studies employing serum obtained from the progeny of each group were conducted. Serum was obtained from five 2 and five 10-day-old birds hatched from eggs collected between 12 and 14 weeks after vaccination and from fifteen 6-day-old birds hatched from eggs collected between 17 and 20 weeks after vaccination. Serum obtained from the 2-day-old chicks was diluted 1:5 with nutrient broth for use in the SN test while all other serum was not diluted.

RESULTS

The endpoints of IL virus titrations (EID_{50}) for eggs obtained from the parent groups are shown in table 2. Although a rather large difference was obtained between the two groups in two instances, such differences were not consistent. The mean of all titrations in the eggs from vaccinated parents was $10^{-5.54}$ while in eggs from unvaccinated parents, it was $10.^{-5.53}$

TABLE II

Results of comparative titration of IL virus in eggs from vaccinated and non-vaccinated parents

Source	EID_{50} -log 10/ml and period of collection				
	4-5-1/2 wks	9-11 wks.	12-14 wks.	14-16 wks.	23-25 wks.
Non-vaccinated	4.83	5.75	5.60	4.80	6.68
Vaccinated	5.17	5.50	6.63	4.77	5.63

The titers (SID_{50}) of IL virus for chicks obtained from the two groups are shown in table 3. Essentially no differences were observed in the titration end points of the two groups.

TABLE III

Results of comparative titration of IL virus in chicks from vaccinated and non-vaccinated parents

Source	SID_{50} -log 10/ml and period of collection	
	4-7 weeks	7-1/2 - 9-1/2 weeks
Non-vaccinated	5.25	4.50
Vaccinated	5.50	4.00

Results of the yolk neutralization tests are shown in table 4. It may be seen that the neutralizing ability of yolk from eggs from vaccinated parents exceeded that of yolk from the non-vaccinated parents. In some instances a considerable

difference existed while in other cases there was pratically no difference. When the data were treated as a whole, the mean neutralization index (NI) for yolk from vaccinated parents equaled $10^{1.20}$ with a standard deviation (SD) of $10^{0.57}$ while the mean NI for yolk from non-vaccinated parents equaled $10^{0.71}$ with a SD of $10.^{0.61}$ The difference in neutralization was significant at the 1% level.

TABLE IV

Results of neutralization tests employing yolk from eggs from IL vaccinated and non-vaccinated parents

	Neutralization indices* (log 10) and time of collection after vaccination						
	1 wk.	2 wks.	3 wks.	4 wks.	8 wks.	12 wks.	14 wks.
Non-vaccinated hens	0.00	1.70	0.00	0.00	0.00	0.88	1.29
	0.00	1.36	1.20	1.20	0.82	0.88	1.28
	0.00	0.00	0.00	0.88	0.00	0.00	1.42
	1.15	0.00	0.00	1.57	0.00	1.00	1.25
	1.03	1.53	1.03	1.03	—	—	0.75
Av.	.44	.92	.45	.94	.20	.69	1.20
Vaccinated hens	1.45	1.21	0.82	0.00	1.03	1.88	1.70
	1.08	1.36	2.37	0.91	0.00	1.38	1.71
	0.00	1.36	1.37	1.45	1.57	1.75	1.92
	1.15	0.00	1.00	1.25	1.03	1.92	1.09
	1.08	0.95	0.82	1.25	1.37	1.00	1.92
Av.	.95	.98	1.28	.97	1.00	1.58	1.67

* Corrected for dilution factor used.

The neutralization indices obtained with serum collected from the progeny of each parent group are shown in table 5. It is again evident that serum obtained from the progeny of vaccinated parents possessed higher neutralizing capacity than did serum from progeny of non-vaccinated parents. When the data obtained with sera from 6-day-old chicks hatched from eggs collected between 17 and 20 weeks after vaccinating were treated as a whole, the mean NI of sera from vaccinated parents equaled $10^{0.46}$ with a SD of $10.^{0.39}$ The mean NI of sera from the non-vaccinated parent's progeny equaled $10^{0.13}$ with a SD of $10.^{0.16}$ Again the difference in neutralization was significant at the 1% level.

DISCUSSION

The results obtained when the IL virus was titered in

TABLE V

Results of neutralization tests employing serum from progeny of IL vaccinated and non-vaccinated parents

	Neutralization indices* (log 10) and time of collection after vaccination and age of chick when bled				
	12-1/2-14 wks. after vaccination		17-20 wks. after vaccination		
	2 days	10 days		6 days	
Non-vaccinated hens	0.82	0.37	0.25	0.50	0.33
	0.95	0.00	0.00	0.00	0.00
	0.00	0.37	0.00	0.00	0.25
	0.00	0.12	0.13	0.17	0.28
	0.00	0.12	0.00	0.00	0.00
Av.	.35	.20		.13	
Vaccinated hens	—	0.25	1.00	0.28	0.67
	0.95	0.12	0.26	0.87	1.30
	0.82	0.58	0.13	0.13	0.33
	0.00	0.00	0.00	0.82	0.50
	0.95	1.12	0.00	0.12	—
Av.	.68	.41		.46	

* Corrected for dilution factor used.

eggs and chicks from the two parental sources indicate that the immune status of the parents under the conditions of this experiment did not markedly influence the ability of the virus to exhibit its typical characteristics. The CAS route of inoculation was used in embryonated eggs in these experiments. As the chorio-allantoic membrane (CAM) route has been shown to produce a higher titer in IL,[4] it is conceivable that the use of this route might offer a more critical tool for evaluating differences in titer in eggs from such parents as used in this trial. In addition, the age of the embryo at time of inoculation may exert an influence.[3] Similarly, it is possible that routes of inoculation other than intrasinus might be more critical in determining viral titers in young chicks.

The data obtained from neutralization studies with yolk and serum collected from the eggs and progeny of the two groups indicate that a higher but not consistent level of neutralizing antibodies occur in the offspring of the vaccinated group in comparison to the non-vaccinated group. The most inconsistant feature encountered in yolk neutralization studies was the presence of occasional yolks from non-vaccinated birds with high neutralizing indices. A similar but infrequent occur-

rence was the complete absence of a neutralizing index in yolk from vaccinated parents. Similar instances were noted in studies on serum from chicks but were less frequent.

With respect to the level of immunity in the parent stock in this experiment, it is conceivable that birds which have suffered an outbreak of the disease or been exposed after vaccination to the virus may have a higher serum antibody level than do vaccinated birds. This in turn may influence the passive immunity in its progeny.

The yolk and sera in these experiments were not heat inactivated prior to use in SN tests. Hitchner, *et al.*[4] have reported that heat inactivation had little influence on serum to be used in the test. However, it is possible that certain nonspecific inhibitors occur in yolk and perhaps in the serum of very young chickens. This aspect will receive further investigation.

While the differences in neutralizing indices between the materials from the two sources are not great in comparison with other diseases such as Newcastle, the magnitude of serum neutralization itself in IL has been reported by Hitchner,*et al.*[4] to be in the range of 10^2 or $10.^3$

In conclusion, it appears that a certain degree of parental immunity does exist in IL of chickens. However, it appears under the conditions of these experiments that such passive immunity as exists is not sufficient to influence homologous viral activity in the embryo or chick hatched from immune parents.

While neutralizing antibodies were detected in yolk and serum of the progeny of immune parents, it is not intended that neutralization tests on such material be considered sufficiently critical or advanced to serve as a means of checking immunity levels. The variation encountered would tend to preclude this type of usage.

SUMMARY

The existence of parental immunity in infectous laryngotracheitis (IL) of chickens was investigated by comparative studies on eggs and chicks from IL vaccinated and non-vacci-

nated parents. The results indicate that yolk obtained from eggs and serum from young chicks from vaccinated parents possess a higher neutralizing capacity than the same materials from non-vaccinated parents. While parental immunity probably exists to a degree, it exhibits little influence on IL viral activity in embryos or young chicks.

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A STUDY IN THE EPIZOOTIOLOGY OF THE CELO VIRUS

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THE chicken-embryo-lethal-orphan (CELO) virus has been found in two contrasting host-parasite situations; one as the causative virus of an acute, often fatal respiratory disease of quail, and the other as the cause of an inapparent infection of chickens. Isolation of the various strains of the virus have been made by Olson,[4] DuBose *et al.*,[2] from quail and by Yates *et al.*,[8] Yates [6] and DuBose[1] from chicken embryos. Serological tests (Yates[6] and Hanson[3]) have indicated that the infection was widespread in chickens, present in turkeys and in some wild birds. Chickens appeared to be the most common host.

Yates and Fry[7] suggested that the CELO virus was sometimes found in the fertile eggs of apparently normal chickens and that the agent was revealed when the normal physiology of the embryonating eggs was altered by inoculation or by some other means. Under normal conditions, however, the endogenous virus was the infection link between generations.

Before the endogenous virus hypothesis can be considered tenable, there must be evidence of some mechanism that allows the fertile egg to carry the virulent virus without evidence of

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infection, at least through the first third of the incubation period. As one of the first steps in the study of this hypothesis, known amounts of the virus were inoculated into fertile eggs before incubation. This procedure was used by Van Roekel *et al.*[5] in the study of the transmission of avian encephalomyelitis.

MATERIALS AND METHODS

Eggs. The fertile eggs used for this study were from White Leghorn hens that were known to carry humoral antibodies against the CELO virus. They were prepared for inoculation by piercing the shell over the air cell.

Virus suspension. Allantoamnionic fluid (AAF) containing the Phelp strain (Yates, 1960) diluted with nutrient broth, was used for these trials. The ELD_{50} titer of this strain in ten or 11-day-old embroys was $10.^{-10}$ The inoculum in these trials consisted of 0.1 ml of the various virus suspensions plus 0.05 ml of the antibiotic suspension.

Trial I. One hundred fifty-five eggs were divided into ten sets of 14 eggs each and one set of 15. The 14 egg sets were inoculated with the various dilutions of the CELO virus from 10^{-1} through 10^{-10}. The 15 egg control set was inoculated with nutrient broth. The suspensions used for inoculation were all treated with streptomycin (33 mg/ml). The 0.15 ml inoculum per egg was administered with a 1.0 ml "tuberculin" syringe fitted with a 0.5 inch 25 gauge needle. After inoculation the holes in the shells were covered with melted paraffin and the eggs were placed in a Jamesway 240K incubator. During the 11th day of incubation, the eggs were removed from the incubator and candled to determine the number of living embryos in each set. Eggs containing living embryos were returned to the incubator. Embryos were observed at least once daily from the 12th to the 19th day. At this time, two embryos from each set were selected and their AAF harvested. The AAF harvested from the two embryos was pooled and used for virus recovery. The rest of the eggs carrying embryos were placed in pedigree boxes and returned to the incubator for hatching.

On the 22nd day, the chicks of each set were examined. Two in each group were killed and a portion of the liver and intestine were harvested for virus reisolation trials. Approximately one gram samples of liver (or intestine) from the two chicks were suspended in 4.5 ml of nutrient broth and ground using a TenBroeck grinder.

The AAF harvested after 19 days of incubation and the suspensions of tissues harvested from the chicks at hatching were used for virus recovery studies. Each sample was carried through three serial, embryo passages. For each passage, a set of five or six, 11-day-old embryos was used. The suspensions of the chick tissues were treated with streptomycin (33 mg/ml) before being used for embryo inoculation.

Trial II. For this trial, 187 eggs were divided into 11 sets of 17 eggs each. The experimental procedure was similar to that of trial I.

RESULTS AND DISCUSSION

The eggs in these trials were inoculated by inserting the needle through the air cell. The inoculum was deposited just beyond the inner shell membrane. This route of inoculation apparently caused no adverse mortality and most embryos, alive after 11 or 12 days, were alive on the 19th day of incubation. The CELO virus was reisolated from many of the samples of AAF harvested from the pair of embryos killed in each set the 19th day of incubation. None of the embryos killed was noticeably dwarfed. In most cases, it was difficult to harvest sufficient AAF from the two embryos to inoculate embryos used for virus recovery. Two and three serial, embryo passages were needed to reisolate the virus from the AAF of eggs receiving the more diluted inocula.

In trial I, none of the embryos receiving the virus diluted 10^{-1} hatched although five were alive on the 20th day and one pipped the shell. In the other dilutions, the livability and hatchability were similar to the control group. Chicks produced from eggs receiving larger amounts of virus showed definite weakness and nervous signs. The usual signs were leg weakness, body vibration and ataxia. The CELO virus was

not reisolated from the samples of liver and intestine harvested at hatching from two chicks in each set.

The results of trial II were much the same as in the first trial, except the virus was not reisolated from AAF of eggs receiving the virus suspensions diluted more than $10.^{-5}$ The CELO virus was reisolated from the liver and intestine samples of the chicks hatched from eggs receiving the virus diluted $10.^{-2}$ Only one chick from the eggs receiving the virus diluted 10^{-4} showed nervous symptoms. A summary of the virus isolation attempts and the condition of the chicks at hatching is presented in tables 1 and 2.

TABLE I

Results of inoculating unincubated eggs with various decimal dilutions of the CELO virus. Trial I.

Dilution of virus	Number embryos alive[1] at		Virus isolations[3] (AAF)	Chicks hatched and condition	
	11 days	20 days		Normal	Nervous signs and weakness
10^{-1}	7	5	2EP	0	0
10^{-2}	11	8	1EP	3	2
10^{-3}	13	11	1EP	5	3
10^{-4}	12	10	1EP	5	4
10^{-5}	11	9	1EP	6	0
10^{-6}	12	10	—	8	1
10^{-7}	12	10	—	7	0
10^{-8}	13	11	2EP	7	0
10^{-9}	12	10	—	7	1[2]
10^{-10}	11	7	3EP	6	0
Controls	13	11	—	9	0

[1] Fourteen eggs were inoculated per dilution, 15 control eggs incubated. AAF harvested from two embryos in each set the 19th day of incubation.

[2] Leg ataxia.

[3] Number of serial embryo passages (EP) needed for virus isolation, starting with AAF harvested at 19th day of incubation.

The virus strain used in these trials had an ELD_{50} titer in ten or 11-day-old embryos of $10.^{-10}$ When inoculated into fertile eggs before incubation, the ELD_{50} titer was approximately $10.^{-1.5}$ This difference in titer must be due to some biological mechanism that was able to "tie-up" the virus and prevent it from destroying the developing embryo. This "tie-up" of the virus may have been due to antibodies, known to be present in the yolk of eggs from hens exposed to the virus. That the virus was present and viable was shown by the virus isolations from AAF harvested the 19th day of incubation.

TABLE II

Results of inoculating unincubated eggs with various decimal dilutions of the CELO virus. Trial II.

Dilution of virus	Number embryos alive[1] at 12 days	Number embryos alive[1] at 20 days	Virus isolation (AAF)	Chicks hatched	Virus isolations[3] (Chicks)
10^{-1}	0	0			
10^{-2}	15	13	2EP	5	3EP
10^{-3}	15	13	1EP	7	—
10^{-4}	16	14	1EP	9[4]	—
10^{-5}	16	14	2EP	8	—
10^{-6}	16	14	—	13	—
10^{-7}	15	13	—	8	—
10^{-8}	16	14	—	11	—
10^{-9}	16	14	—	8	—
10^{-10}	17	15	—	13	—
Controls	17	14	—	9	—

[1] Seventeen eggs were inoculated per dilution. AAF harvested from two embryos in each set the 19th day of incubation.
[2] Number of serial embryo passages (EP) needed for virus isolation, starting with AAF harvested, from two embryos the 19th day of incubation.
[3] Number of serial embryo passages needed for virus isolation, starting with tissue samples from 2, one-day-old chicks.
[4] Nervous manifestations in one chick.

Yates[6] reported holding samples of CELO virus for 20 days at 37 C with a titer drop of approximately two logs.

The study supports the endogenous virus hypothesis by giving evidence of a biological mechanism that allowed the embryo pathogenic virus to be carried in embryonating eggs without pernicious effect, except in high concentrations. It also demonstrates the usefulness of a technique in investigating methods of revealing inapparent viruses of embryonating eggs and in the resulting progeny.

SUMMARY

The CELO virus was found to be relatively non-pathogenic to the developing chicken embryo when introduced before incubation.

The virus was reisolated several times from allantoamnionic fluid harvested the 19th day of incubation; it was also reisolated on one occasion from intestine and liver tissue harvested from one-day-old chicks.

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SEROLOGICAL STUDIES OF AVIAN PLEUROPNEUMONIA-LIKE ORGANISMS (PPLO) WITH EDWARD'S TECHNIQUE*

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EDWARD and Fitzgerald[6] observed that the growth of PPLO colonies could be inhibited by the incorporation

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of specific hyperimmune antisera, produced in rabbits, in appropriate solid media. These studies deal with attempts to utilize this phenomenon in the isolation and identification of avian PPLO. In preliminary experiments, we were unable to demonstrate inhibition of colony growth of PPLO with homologous chicken antiserum, from birds infected via the respiratory tract. This lack of success confirmed Edward's results and indicated that only hyperimmune rabbit sera were capable of producing this colony inhibition effect.

MATERIALS AND METHODS

Antigens were prepared from isolates which were believed to be pure strains of PPLO because of their uniform and consistent maintenance of such characteristics as colony type, growth rate and fermentation reactions over a long period of observation and through many (50 to 150) passages in culture media. The antigens were made by growing the PPLO in 1000 ml batches of PPLO broth without crystal violet (Difco) to which was added 10 percent horse serum. After six days of incubation, the organisms were separted out and washed three times in buffered physiological saline (pH 7.6) with a Servall angle centrifuge. Each centrifugation lasted 30 minutes at 15,000 rpm (27,000G). After washing, each of the antigens was resuspended in 50 ml of buffered saline and used at this concentration to produce antisera in rabbits. The densities of the antigen suspensions for each of the PPLO isolates varied very widely. Some were slightly cloudy, others quite dense.

The rabbits were given a series of intraperitoneal injections, arbitrarily decided at 1 ml per injection; three injections weekly for the first 2 weeks and one injection weekly thereafter. With this type of antigen and course of injections, a period of from 3 to 4 months was required to produce a good, colony inhibiting antiserum. Control sera collected from the rabbits at the start of the course of immunization was shown to be non-inhibitory.

The colony growth inhibition test was conducted by spreading a loopful (0.02 ml) of a profusely growing broth

culture of PPLO over an area of approximately 15 mm diameter of a segment of an agar plate medium in which 5 percent of rabbit antiserum was incorporated. The basal medium consisted of PPLO agar (Difco) to which was added 10 percent swine serum and penicillin and thallium acetate as inhibitors. All plates were incubated in candle jars and examined with a dissecting microscope after 6 days of incubation.

RESULTS

As described by Edward and Fitzgerald,[6] inhibition of colonial growth was observed on plates containing specific antiserum, while profuse growth occurred on plates containing normal rabbit serum, non-specific rabbit antiserum or no rabbit serum. A sample protocol of a series of colony growth inhibition tests is presented in table 1. Up to the present time, six different specific antisera have been prepared.

TABLE I

A sample protocol of a series of colony growth inhibition tests

PPLO culture	Hyperimmune rabbit serums used							
	None	293	ATU	C26	K18B	8	C2	ASA
293	3*	0	3	3	3	3	3	3
ATU	3	2	0	2	2	2	3	2
C26	3	2	3	0	2	2	3	2
K18B	3	3	3	3	0	3	0	3
8	3	2	3	2	2	0	3	3
C2	3	3	3	2	0	2	0	2
ASA	3	2	3	3	3	3	3	0
WJB	2	0	3	3	2	2	2	2
PH	3	0	3	3	3	3	2	2
H695	3	3	3	3	3	3	3	3
H1344	3	0	3	3	3	3	3	3
113R	2	2	0	2	2	2	2	2
4F	2	2	2	0	2	2	2	2
C126	2	2	2	2	2	0	2	2
114R	2	2	2	2	2	2	2	2

* 3 = colonies too numerous to count
2 = 10 to 100 colonies
1 = 1 to 10 colonies
0 = no colonies

On the basis of tests with these antisera, 170 cultures of PPLO obtained from other laboratories and by isolations in this laboratory were identified. These isolates fell into six distinct groups and another group containing unclassified cultures: S_6 group contained 81 isolates, C group-27 isolates,

R39A group-11 isolates, SLO group-10 isolates, K18B group-17 isolates, SA group-1 isolate, unclassified-23 isolates. The origin of some of the previously described isolates which were classified by this procedure is presented in table 5. Information already available indicates that the unclassified isolates fall into at least four different groups (see table 3). While further definitive classification of these isolates awaits the production of additional rabbit antisera, a preliminary classification based on the information already available is presented in table 3.

In the use and interpretation of this test, every effort must be made to insure that the PPLO isolates are pure. In these experiments, some isolates were found that were not inhibited by any of the antisera when first tested. However, retesting of some of the same isolates after purification by repeated single colony picking did produce specific inhibition with a single antiserum and allowed their classification. All of these mixed isolates were of the "non-pathogenic" type which produced large and rapidly growing colonies on PPLO agar containing 10 percent horse serum and 1 percent yeast hydrolysate. On the other hand, up to this time, all isolates which consisted entirely of slow growing organisms producing small colonies on the same medium proved to be pure S_6 type cultures.

In these tests with 5 percent rabbit antisera in the media, only complete inhibition of an undiluted culture was considered specific. Edward and Fitzgerald[6] and Edward and Kanarek[7] indicate that in some cases, complete inhibition may take place only with diluted cultures. Further clarification of the use and interpretation of such incomplete inhibition reactions must be made.

An experiment was designed to see if it was necessary to use 5 percent rabbit antisera to secure colony inhibition since such sera were hard to produce and limited in quantity. Eighteen PPLO isolates (all S_6 type) were inoculated onto a series of plates containing 5, 1, 0.5 and 0.05 percent respectively of S_6 rabbit antiserum and control plates containing no

TABLE II

Characteristics of some PPLO strains not yet classified by the colony growth inhibition technique

Group	Activity in carbohydrates	Growth rate and colony type	Comments
N	—	Rapid growth, large colonies	Groups N and O have already been shown to be serologically distinct on the basis of agglutination reactions by Adler *et al.*, and Kleckner
O	—	Rapid growth, large colonies	Corersponds to *Mycoplasma iners* (Edward & Kanarek)
Infectious synovitis derived	+	Slow growth, small colonies	Grown only on media containing diphosphopyridine nucleotide (DPN) (Chalquest and Fabricant)
R49	+	Rapid growth, large colonies	

TABLE III

Preliminary classification of avian PPLO

Author's classification				Characteristics			
Fabricant	Adler et al.	Kleckner	Edward and Kanarek	Specific inhibiting antiserum prepared	Pathogenicity for birds	Growth rate and colony size	Dextose fermentation
S_6	S_6	A	*Mycoplasma gallisepticum*	+	+	SS*	+
C	C	C	15804	+	—	RL**	+
R39A		D		+	?	RL	+
SLO		E		+	—	RL	—
K18B		B	*Mycoplasma gallinarum*	+	—	RL	—
		F					
SA	SA	G	Mycoplasma	+	—	RL	+
O	O		*iners*	ND	—	RL	—
N	N	H		ND	?	RL	—
Infectious synovitis derived †				ND	+	SS	+
R49				ND	?	RL	+

† Requires DPN
* SS = slow growth rate, small colonies
**RL = rapid growth rate, large colonies
ND = not done

rabbit antiserum. The results of this experiment are presented in table 4. It can be seen that complete inhibition of all 18 isolates only occurred in the plates containing 5 percent rabbit antiserum produced under our conditions.

A preliminary classification of the types of avian PPLO identified up to the present time is presented in table 5. This tentative grouping is still incomplete since further work must be done on the serology and other characteristics of some of the suggested groups.

TABLE IV

The effect of varying dilutions of S_6 rabbit antiserum on inhibition of colonial growth of 18 different isolates of S_6 type PPLO

	S_6 rabbit antiserum in media				
	%	5%	1%	0.5%	0.05%
Complete inhibition		18*	10	4	
Questionable			3	3	
No inhibition	18		5	11	18

* Numbers represent number of cultures out of 18 tried.

DISCUSSION

The main purpose of this study was to find further means for identification of avian PPLO and for the determination of the purity of PPLO cultures. The completeness of inhibition observed in these tests is a valuable indication that pure cultures have been obtained. The fact that it is possible to inoculate as many as 10^6 organisms onto a segment of a plate containing specific antiserum without the growth of a single colony suggests that we have a useful test for such a determination of strain purity.

Our experience corroborates the observations of Edward and Fitzgerald[6] that a long period of hyperimmunization is often necessary to produce good growth inhibiting antisera in rabbits. The lack of satisfactory inhibition observed by Yamamoto and Adler[17] with avian strains and by Olson *et al.*[14] with bovine strains may be attributed to too short a course of hyperimmunization. However, it is possible that satisfactory antisera could be produced in a shorter time with fewer

TABLE V

Classification of some avian PPLO isolates described in previous publications by the colony growth inhibition technique

Isolate designation	Other designation	Sources and reference		Classification
S.		California	Adler *et al.*[2]*	S.
F		California	Yamamoto and Adler[16,17]	S.
SV		California	Yamamoto and Adler[16,17]	S.
C		California	Adler *et al.*[2]	C
TU		California	Yamamoto and Adler[16,17]	C
SA		California	Adler *et al.*[2]	SA
O		California	Adler *et al.*[2]	Unclassified
N		California	Adler *et al.*[2]	Unclassified
PH		California	Adler and Berg[1]	S.
VR		Georgia	Kleckner[11]	S.
DIVA		Georgia	Kleckner[11]	C
CRDA		Georgia	Kleckner[11]	C
54-537		Georgia	Kleckner[11]	K18B
NY		Georgia	Kleckner[11]	R39A
WVU1791	10	West Virginia	Olson *et al.*[13]	S.
699		Eli Lilly & Co.	Ose *et al.*[15]	S.
T1		Texas	Moore *et al.*[12]	S.
C1		Texas	Moore *et al.*[12]	S.
C5		Texas	Moore *et al.*[12]	Unclassified
C6		Texas	Moore *et al.*[12]	S.
C7		Texas	Moore *et al.*[12]	R39A
C8		Texas	Moore *et al.*[12]	R39A
W	Winchester	Virginia	Gross[9]	S.
DPR-2		Georgia	Kleckner[11]	SLO
293		New York	Calnek and Levine[3] Fabricant *et al*[5]	S.
A5969	Connecticut antigen strain	Connecticut via	Jungherr *et al.*[10]	S.
Mycoplasma gallinarum	PG16	Massachusetts via	Edward and Kanarek[7]	K18B
Mycoplasma gallisepticum	PG31	Massachusetts	Edward and Kanarek[7]	S.

* Superscripts refer to references

inoculations with a more concentrated antigen. The problem of the production of growth inhibiting antiserum needs further study. Our data do not confirm the suggestion by Edward and Kanarek[7] that antigens for the immunization of rabbits should be grown on media containing rabbit serum. We were able to produce highly specific and satisfactory antisera from antigens grown on media containing horse serum.

The results of the studies done so far—even though far from completed—suggest that there is a good correspondence in the results of classification of avian PPLO on the basis of either the growth inhibition or the agglutination tests. However, the growth inhibition test has some advantages: 1. It makes it possible to identify isolates that do not produce a satisfactory agglutinating antigen. 2. It allows the identification of the purity of the isolates tested. 3. It avoids the cross reactions often noted in the agglutination test (Kleckner[11]). These cross reactions may be due to the presence of mixed cultures in some of the agglutinating antigens. 4. Once a satisfactory antiserum is made, it may be used to test a large number of isolates without the necessity of producing an antigen for each isolate.

Experience with avian PPLO suggests that classification should await the availability of complete information on the following characteristics: serology, pathogenicity for birds, colony size and growth rate, ability to ferment carbohydrates, special nutritional requirements. These characteristics seem to be more useful in the study of avian PPLO than Edward's criteria of the production of "film" or "spots" on the media and the hemolysis of blood agar.

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SUMMARY

Edward and Fitzgerald[6] observed that the growth of PPLO colonies could be inhibited by the incorporation of specific hyperimmune rabbit antisera in appropriate solid media. With some modification of this technique, 170 cultures of PPLO, obtained from other laboratories or by isolation in this laboratory, were identified. These isolates fell into six different groups and another group containing unclassified cultures: S_6 group contained 81 isolates, C group 27 isolates, R39A group 11 isolates, SLO group 10 isolates, K18B group 17 isolates, SA group 1 isolate, unclassified 23 isolates. Based on these findings and other information available in the literature, a preliminary classification of the presently recognized avian PPLO is suggested. All 81 of the isolates which were identified at this laboratory as pathogenic (S_6 type) isolates on the basis of cultural characteristics fell into a single serological group confirming similar observations made by Kleckner who used agglutination reactions.

A high degree of specificity and complete colonial inhibition was observed in isolates which had been carefully selected on the basis of uniformity of cultural characteristics. This procedure might be a useful tool for the isolation of avian PPLO from mixed cultures and for the establishment of the purity of PPLO isolates.

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PLEUROPNEUMONIA-LIKE ORGANISMS ASSOCIATED WITH SYNOVITIS IN FOWLS*†

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THERE are many types of arthritic diseases which affect turkeys and chickens. Although the majority of them are caused by bacteria, the most common one, infectious synovitis (IS), is not caused by any of these known organisms. Several workers[4,5,11,13,17] have studied the nature of the IS agent and have concluded that it was distinct from any previously described agent. It was presumed to be a large virus or rickettsia as suggested by Lecce *et al.*[11]until Lecce[10] reported the isolation of a pleuropneumonia-like organism (PPLO) from allantoic fluid of infected embryos. The purpose of this study was to isolate and identify the agent of infectious synovitis.

MATERIALS AND METHODS

All chicken hatching eggs and chicks used in this study were obtained from the veterinary college poultry disease research farm flock of White Leghorns which did not have any respiratory infection during this study. The flock had been vaccinated only for infectious bronchitis. Repeated attempts to isolate PPLO from the birds or their eggs failed. Periodic agglutination tests of these birds have never resulted in any positive reactions for PPLO. The turkey poults used in this study were Empire Whites obtained from a com-

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mercial hatchery. Neither the original breeding flock nor the poults that were inoculated in the various trials were known to have undergone any respiratory disease during this study. All birds experimentally inoculated in this study were held in modified Horsfall units to provide complete isolation for each group inoculated.

For the most part, this study was conducted with four different isolates of the agent that were obtained from other workers. These included isolates 1853 from Dr. N. O. Olson, the Maine and Texas isolates from Dr. K. E. Price, and the 1071 isolate from Dr. J. G. Lecce. Isolate 298 obtained from Dr. G. H. Snoeyenbos as well as some recent field isolates obtained at this laboratory were also included in some of the trials.

All broth media contained 1,000 units of penicillin and a 1 to 1,000 concentration of thallium acetate per ml as inhibitors. Unless otherwise indicated, all solid media contained the same level of penicillin and 1 to 2,000 concentration of thallium acetate. All plates were incubated in the candle jar for 6 days as described by Fabricant,[7] and all broth media were incubated aerobically. Only sera which had been Seitz filtered and frozen were used in this study. In addition, all sera added to media containing beta-diphosphopyridine nucleotide (DPN) were heat inactivated at 56 C for 30 minutes.

Swine infusion broth was prepared in a similar manner to the chicken infusion media described by Hofstad and Doerr.[9] However, all the ingredients of this media were of porcine rather than avian origin. Twenty percent swine serum was added to the broth as well as the usual inhibitors.

Beta-diphosphopyridine nucleotide* (DPN) powder was dissolved in sufficient distilled water to make a 1 percent solution and filtered through a Seitz pad. The sterile filtrate was transferred in 5 or 10 ml quantities to screw cap vials and kept at 0 C. The quantity desired was thawed and added to the broth media with a sterile pipette just prior to incubation of the media or added to the solid media just before the plates were prepared. The unused portion was always discarded. All

* Sigma Chemical Co., St. Louis, Missouri.

solid media prepared with DPN were stored at 5 C. However, only plates which had been stored for less than 7 days at this temperature were used in this study.

L-cysteine (free base)* was always dissolved in distilled water as a 1 percent solution, filtered through a Seitz pad, and immediately added to the media. When freshly prepared in this manner it is a clear solution. When a white precipitate formed after approximately 48 hours at 5 C or after freezing, the 1 percent solutions were discarded.

RESULTS

Attempts to isolate a bacterium or a PPLO. Each yolk, allantoic fluid, ground up suspension of liver and spleen, and synovial fluid from embryos or birds inoculated with four different isolates (1853, Maine, Texas, and 1071) was seeded on blood agar plates, meat piece media or liver-brain media at least three times. All culture attempts were negative for bacteria after 72 hours of aerobic or anaerobic incubation.

Attempts to isolate a PPLO from infectious yolk, allantoic fluid, or synovial fluid from the 1853, Maine, Texas, and 1071 isolates by inoculating Adler's overlay media (Adler *et al.*[1]) were unsuccessful. Lecce[10] reported that PPLO were isolated from infectious allantoic fluid that was inoculated onto PPLO agar containing 10 percent swine serum. This growth only occurred as satellite colonies around a streak of *Staphylococcus aureus.* The plates had to be incubated in a candle jar for the best results. We obtained similar colonies from three laboratory isolates of the synovitis agent (1853, Maine and Texas) as well as the 1071 isolate by Lecce's technique. These colonies, designated infectious synovitis derived (ISD) colonies, were very small and only grew very close to the *Staph. aureus* streak (figure 1). Occasionally, a few of these colonies had a nipple and appeared to be a PPLO as Lecce described.

Four isolates of *Staph. aureus* in addition to the one Lecce had used did not produce these satellite colonies by themselves. Three of them aided the ISD colonies to grow from infectious

* Nutritional Biochemicals Corp., Cleveland, Ohio.

allantoic fluid. Addtional trials also indicated that the candle jar incubation of the plates was superior to aerobic incubation and that horse serum could not be substituted successfully for swine serum in this media. Over a period of time, 52 uninoculated embryos from 8 to 16 days of age were cultured by Lecce's technique. No isolation of any satellite colonies around the *Staph. aureus* streak were ever obtained.

Thallium acetate at a concentration of 1 to 1,000 and mycostatin* at 100 or 500 units per ml did not alter the pathogenicity of four different isolates (1853, Maine, Texas, and 1071) for embryos. Furthermore, thallium acetate at a concentration of 1 to 2,200 in the solid media permitted satisfactory growth of the *Staph. aureus* and the ISD colonies.

Culture media with beta-diphosphopyridine nucleotide for cultivation of the agent. In an effort to eliminate the Staphylococcus, 87 different liquid and solid media were devised and tested for growing the ISD colonies. None were suitable until DPN and cysteine were added to the media. Lwoff and Lwoff[12] had established that the "V" factor necessary for the growth of Hemophilus, an organism that also exhibited satellitism to *Staph. aureus,* was actually coenzyme I and later known to be diphosphopyridine nucleotide (DPN). Snyder and Broh-Kohn[16] reported that cysteine could be substituted for the second or "X" factor required for growth of Hemophilus. Therefore, a PPLO agar was prepared which contained both 0.1 percent DPN and 0.1 percent cysteine in addition to the 10 percent heat inactivated, swine serum and thallium acetate and penicillin. All of the constituents were added to the melted PPLO agar after it had cooled to approximately 45 C. This media, designated 1308P, was compared with the plates which incorporated the *Staph. aureus* streak.

Six-day-old embryos were inoculated with yolk material of a single isolate of the IS agent via the yolk sac. A loopful of the allantoic fluid from each of three embryos which died on the seventh day postinoculation were separately inoculated onto both 1308P and *Staph. aureus* streak plates. The plates were incubated in the candle jar for 6 days. Between

* E. R. Squibb & Sons, New York, New York.

10 and 25 colonies were obtained on both types of plates that had been inoculated with the material from two of the eggs. The allantoic fluid from the third egg yielded less than 10 colonies on the 1308P but no growth on the *Staph. aureus* streak plate. When agar blocks containing a few of the ISD colonies on the 1308P were transferred to a similar sterile plate, very heavy growth of nippled colonies, resembling PPLO colonies ,were obtained after 6 days incubation in the candle jar. Three such plate to plate transfers were made successfully without any apparent loss in the amount of growth. This was the first media which supported the growth of the ISD colonies without the *Staph. aureus* streak.

The next series of experiments were designed to determine if this media would support the growth of other isolates of the agent and to test some variations in this media. Infectious allantoic fluid from four different isolates of the IS agent (1853, Maine, Texas, and 1071) as well as allantoic fluid from uninoculated eggs were cultured on several variations of the 1308P media. In addition, the first and third broth passages of these same allantoic fluid inoculums were also subcultured onto each of these types of solid media.

The results of these trials indicated that a solid medium (1396P) which contained 0.1 percent DPN but no cysteine, was superior to 1308P for original isolation from infectious allantoic fluid. However, both media yielded equal amounts of luxuriant growth from either of the broth passages. Solid media which contained either 0.05 or 0.01 percent DPN and no cysteine, yielded good growth but the colonies were more roughened on the edges, not as clearly nippled, and somewhat smaller and flatter than those obtained on the media which contained 0.1 percent DPN. The following types of media failed to support growth of ISD colonies: media with no DPN, no serum or serum substitute, 0.1 percent niacinamide instead of DPN, DPN and 0.1 percent cysteine hydrochloride, 1 percent serum fraction (Difco) or 10 percent horse serum instead of swine serum. In additon, it was again noted that the incubation of these plates in the candle jar gave better results than incubation aerobically.

Several different broth media were tested by seeding them with different types of inoculums. These included agar blocks containing colonies, allantoic fluid, yolk, and synovial fluid. Approximately 3 ml of broth media were inoculated with 0.2 ml of infectious material. The infectivity of the inoculum was determined by inoculating the same material into 4 to 6-week-old chicks.

The results of these trials indicated that medium 1308B which contained PPLO broth without crystal violet + 0.1 percent DPN + 0.1 percent cysteine + 10 percent heat inactivated swine serum + inhibitors was satisfactory. Some growth of these organisms was also obtained in media with 0.05 percent DPN and 0.05 percent cysteine as well as in media with 0.1 percent DPN and 0.05 percent cysteine. However, broth media with DPN but no cysteine did not support growth of these organisms.

In two trials, yolk and allantoic fluid from the same infected embryo were cultured separately in media containing DPN and cysteine. Both trials included infectious material from five different isolates of the agent as well as yolk and allantoic fluid from uninoculated embryos. Luxuriant growth was obtained from all the allantoic fluid inoculums but the control group. On the other hand, very few colonies or no growth at all was obtained from the yolk of the same eggs. The allantoic fluid was superior to the yolk for the isolation of this agent from infected embryos.

Although the swine infusion broth did not support the growth of the infectious synovitis agent, it served very well for the growth of S_6 type PPLO. Additional experiments to determine the validity of these observations are in progress.

Infectious synovitis derived (ISD) PPLO isolations from field cases of synovitis. Throughout the course of this study, attempts were made to isolate the agent from field cases of the disease on artificial media. Material from different cases was usually cultured by inoculating some of the synovial fluid from the affected joints directly into various types of broth media. A few of these cases were also cultured in embryos. Most of these cases were investigated before a satisfactory

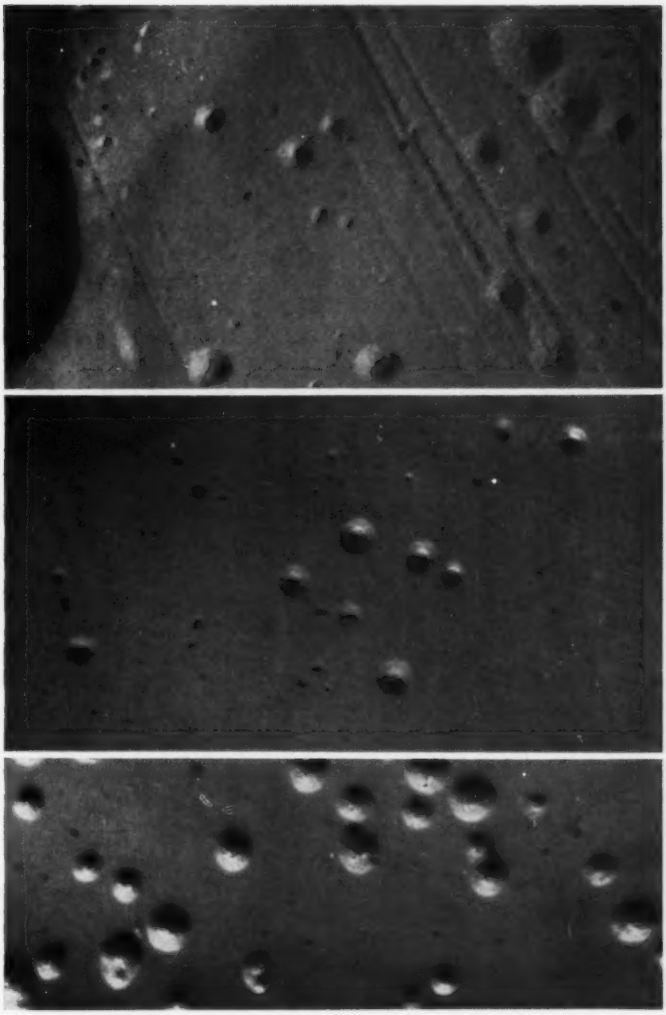


Fig. 1 (top). Colonies of NTF isolate (infectious synovitis derived) growing as satellites to *Staphylococcus aucreus* shown at left. (×150).

Fig. 2 (middle). Colonies of NTF isolate on DPN agar without staphylococcus (×150).

Fig. 3 (bottom). Colonies of standard S_6 type PPLO on swine serum PPLO agar (×150).

broth media was developed. However, two ISD PPLO isolations were made.

Accession EA 10808 (*isolate 1472*). No history was submitted with these 7-week-old broilers. Three of the birds in this accesion had keel bursitis and enlarged hocks. The swollen joints were filled with excess, mucoid exudate. The spleens of all the birds were also enlarged. Diluted synovial fluid from these birds was inoculated into embryos via the yolk sac and isolations of PPLO colonies were obtained from the allantoic fluid of these embryos only on media containing DPN. Cultures of the same allantoic fluid in SA (horse blood, agar slant + overlay of PPLO broth containing 10 percent swine serum) did not yield any growth. This media, as described by Fabricant,[7] is satisfactory for the cultivation of both pathogenic and nonpathogenic type PPLO. The growth of this isolate was not inhibited by S_6 type antiserum in the plate inhibition test of Edwards and Fitzgerald.[6] Since this isolate failed to grow after a few serial passages in artificial media, no pathogenicity trials were conducted.

Accession 60142 (*isolate NTF*). Several birds in a flock of 4500, 10-week-old White Holland turkeys were noted to be lame and a few had swollen hocks. This lameness had been observed for about 2 weeks when the birds were submitted for diagnosis. The flock as a whole appeared in good health and there were no respiratory symptoms noted. The affected birds had excess synovial exudate which contained some caseous material along the tendon sheaths in the shanks and in the hocks. The caseous exudate varied in color from white to yellow. The liver and spleen appeared normal. There were no gross lesions in the respiratory tract of any of the birds submitted. The liver was cultured on blood agar which was incubated aerobically. The synovial fluid was cultured in the same manner and also in 985 broth media. This media consisted of an agar slant (PPLO agar + 20 percent swine serum and 1 percent Supplement B (Difco) and an overlay broth (swine infusion broth + 20 percent swine serum + inhibitors). The blood agar cultures of both the liver and the syno-

vial exudate yielded many colonies of *Staphylococcus epidermis*. No isolations were obtained in three broth passages of the 985 media.

Synovial fluid was obtained with three sterile cotton swabs by saturating them with this fluid and inoculating all three swabs into 5 ml of sterile nutrient broth. The diluted synovial fluid wtih 40,000 units of penicillin added per ml was inoculated into 5-day-old embryos. Of the seven eggs that were inoculated, one died on the 2nd day, two on the 6th day, two on the 8th day, and one on the 9th day postinoculation. Both allantoic fluid and the yolk of one embryo that died on the 8th day postinoculation were cultured in 1308B broth media. The first broth passage after 3 days incubation was transferred to 1308P. Approximately 50 typical PPLO colonies were obtained from the allantoic fluid but none from the yolk. An agar block containing several of these colonies was transferred to a tube of broth media which contained DPN and cysteine. After 3 days of incubation, transfers were made to both SP and 1396P plates. There was no growth on the SP plate, but heavy growth was present on the 1396P. This broth passage was serially transferred in the same media and inoculated into chicks and poults to determine its pathogenicity. This trial is described later in this report. A broth culture of this isolate was also tested by the plate inhibition test of Edward and Fitzgerald[6] with S_6 type antiserum. The growth of this isolate was not inhibited by the S_6 type antiserum indicating that it, like the 1472 isolate, was not an S_6 type PPLO.

Seven additional attempts were made to isolate a PPLO from birds that had no respiratory tract lesions but did have lesions of synovitis. Each of these cases were cultured in various broth media but no isolations of any PPLO were obtained. Unfortunately, only three of these cases were cultured in a media containing DPN. The reason for the negative results in these cases was impossible to determine.

Some characteristics of infectious synovitis derived (ISD) PPLO. 1. Morphology. The colonies observed on 1308P (basal agar + DPN + cysteine) or 1396P (basal agar +

DPN) were similar in shape and size to an S_6 or pathogenic type PPLO as described by Adler *et al*[2] (figures 2 and 3). The colonies originally isolated from synovial fluid and for the first few passages in artificial media were uniformly quite small and discrete. However, after several passages in artificial media, distinct variation in colony size as well as a definite tendency to form multiple colonies was observed. A gray, crystalline-like film usually was present on the surface of the plate after 6 days of incubation whenever heavy growth was obtained. This film was particularly noticeable if the plate was held for an additional 1 or 2 days at room temeprature following the usual incubation period. The colonies when fixed with Bouin's solution and stained with Giemsa stain also resembled an S_6 type pathogenic PPLO.

To determine the morphology of these organisms in broth culture, stained preparations were made of four different isolates as well as of uninoculated but incubated media. A loop of the various broth cultures was transferred to a clean glass slide, gently dried by heat, and then fixed with Bouin's solution for 15 minutes. After rinsing the Bouin's solution off with distilled water, the preparation was stained for 30 minutes with one to 10 dilution of freshly filtered Giemsa stain. Each of the cultures was stained by this technique after 24, 48, and 72 hours of incubation. Again, the morphology of these organisms was similar to S_6 pathogenic type PPLO as described by Yamamoto and Adler.[18]

2. Biochemical reactions. Cultures of all five laboratory isolates (1853, Maine, Texas, 1071, and 298) as well as two isolates from field cases (1472 and NTF) were inoculated into carbohydrate media. These media consisted of basal broth + 0.05 percent DPN + 0.05 percent cysteine, to which was added 0.018 grams of phenol red per liter and one percent of either dextrose, lactose, maltose, sucrose, or mannite. Two separate trials were conducted and in each trial uninoculated tubes of each sugar media were included as controls. All tubes were incubated aerobically for 21 days before the final readings were taken. In both trials, all of the isolates fermented both dextrose and maltose. In every case, the media had

changed color after 3 or 4 days of incubation and no further changes were noted in any of the tubes after this time. There was no fermentation by any of the strains of the other three sugars.

3. Serological tests. Broth cultures of ISD PPLO isolates 1853, Maine, Texas, 298, NTF, and 1472 were inoculated on plates in which were incorporated 5 percent hyperimmune rabbit sera prepared against a standard S_6 type PPLO (293), or one of five non-pathogenic types of PPLO. The technique was described by Edwards and Fitzgerald,[6] and by Fabricant.[8] None of these plates inhibited the growth of the NTF isolate, an indication that it belonged to a different serological type. Similarly, the plates that contained the S_6 type antiserum did not inhibit the growth of any of the other ISD PPLO isolates.

4. Growth on non-inhibitory media. In an attempt to determine if the inhibitors normally present in the media had altered the appearance of these organisms, six isolates (1853, Maine, Texas, 1071, 298, and NTF) were inoculated on solid media without inhibitors. Agar blocks containing several colonies were taken from plates which contained penicillin and thallium acetate and inoculated into broth media 1491B (0.5 percent DPN + 0.5 percent cysteine) which also contained these inhibitors. After 3 days of incubation, transfers were made to plates which contained all the ingredients of 1308P (DPN + cysteine) media but with no thallium acetate or penicillin. These plates were then incubated in the candle jar for 3 days. Heavy growth was noted at this time and an agar block with several colonies was smeared onto another plate of the same media. Five such plate to plate transfers were attempted. However, because of mold contamination on some of the plates, four of the isolates were discarded at various passages earlier than the fifth. Only two of the isolates 298 and NTF) were successfully carried for the full five passages. When stained with Giemsa, the colonies obtained on the fifth passage appeared similar to the colonies that had grown on the first passage. Neither of these isolates developed any visible changes in the gross morphology of the colonies. This was also true of the other isolates, after having

been transferred only for two or three passages on non-inhibitory media. These data indicate that the organisms which were recovered from material containing the IS agent were a PPLO of a new type.

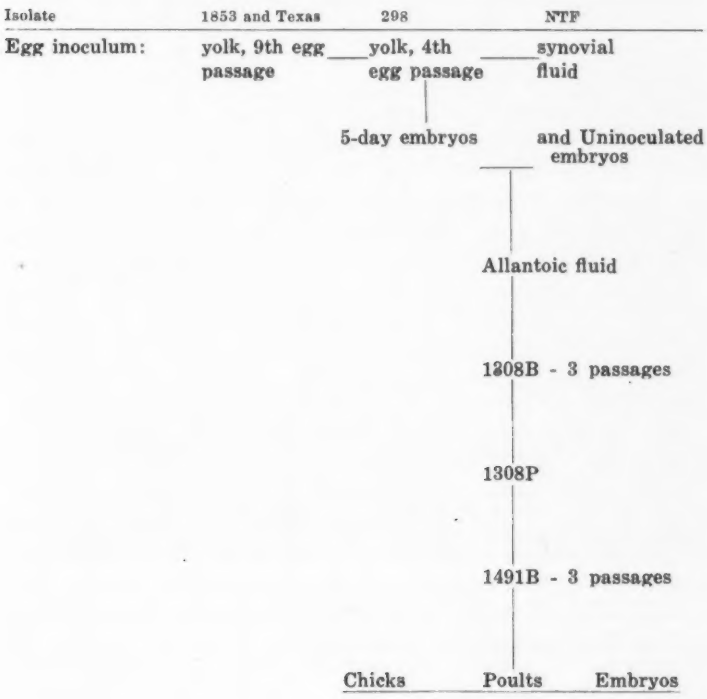
The pathogenicity of PPLO, infectious sinusitis derived (ISD). 1. The first trial was conducted to determine if the satellite colonies to the *Staph. aureus* streak on Lecce's medium were pathogenic for birds. Intravenous inoculation into 4-week-old chicks of these ISD PPLO colonies from the 1071 isolate produced signs and lesions typical of birds affected with IS. Because these colonies were obtained from the first passage on artificial media, other agents that were not visible could have been present in the inoculum.

2. The design of the second experiment is given below. Five-day-old embryos were inoculated wtih infectious yolk of the four different isolates. The inoculum for the fourth group (NTF) was a diluted, synovial exudate obtained from the turkeys presented for diagnosis. A control group of uninoculated eggs was also included in this trial.

Allantoic fluid was harvested from embryos which died in each group except the control group which were still alive. Two-tenths ml of this fluid from each group were inoculated into separate tubes of 1308B media. Three serial broth to broth passages were made after a three day incubation period. The first and the third of these broth passages were also tranferred to 1308P and good growth of typical PPLO colonies were obtained from each of the different isolates. Agar blocks containing several colonies from the third broth passages were transferred to 1491B broth. Three more serial broth to broth transfers were made with a resulting good growth. No growth resulted from the controls. The last broth culture which represented the seventh passage on artificial media of the original allantoic fluid, was inoculated into chicks, poults, and 5-day-old embryos.

The inoculum for the chicks and poults was a 1 to 5 dilution of the broth culture with sterile nutrient broth as the diluent. Both the chicks and the poults were 4 weeks old and

five of each were inoculated in each group including the control groups. Each banded bird was given one ml of this diluted broth culture intravenously. Just prior to inoculation with the broth culture, a blood sample was taken from each bird and identified. The serum from each of these samples was later tested with commercial PPLO antigen by the plate agglutination test. As usual, each group of birds was kept in separate, modified Horsfall units throughout the experiment.



The postmortem findings of the chicks and poults inoculated with the NTF isolate are summarized in table 1. The chicks that had been inoculated with the NTF isolate had a green diarrhea which began on the 8th day postinoculation. At this same time, the birds were noted to be very inactive and droopy. On the ninth day following inoculation, one of these chicks was bled and necropsied. This bird had a severely swollen liver and spleen as compared with that of a control Both hocks of this bird from the NTF group were slightly swollen. These areas contained an excessive amount of typical mucoid exudate and a few flecks of yellow caseous material. The other four birds in this group were all bled and necropsied on the 21st day postinoculation. At this time, all four were noted to have severely swollen foot pads and keel bursitis. Internally, these birds had normal appearing spleens but the livers looked slightly swollen. The hock joints and foot pads of each birds were filled with typical mucoid exudate and a little yellow caseous exudate. There were no grossly visible lesions in the respiratory tracts of any of these birds.

Histological sections of the liver of the first NTF bird that was killed showed a very marked focal accumulation of reticular cells in the perivascular and sinusoidal areas (figures 4 and 5). The other birds from this same group also had a few of these areas, but not as many as the first bird. This lesion in the liver is considered to be a distinctive characteristic of infection with IS agent.

Cultures were taken from the bird killed on the 9th day and from two of the four birds killed on the 21st day following inoculation. Similar samples were obtained from the same number of birds in the control group at the same time. The synovial fluid from each of these birds was inoculated directly onto a blood agar plate which was incubated aerobically for 72 hours. A swab of the synovial fluid was also inoculated into 1565B media (0.1 percent DPN + 0.05 percent cysteine). After 72 hours of aerobic incubation, transfers were made to 1396P. There was no evidence of any growth on any of the

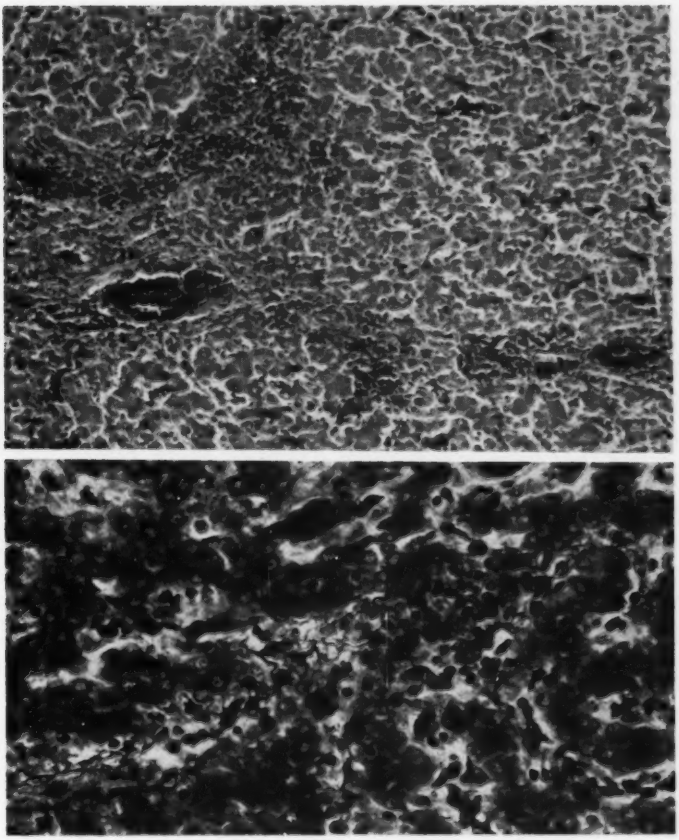


Fig. 4 (top). Section of liver of infected chick with accumulations of reticular cells in perivascular and sinusoidal areas. Hematoxylin and eosin stain (×160).

Fig. 5. (bottom). Section of liver of infected chick showing reticular network. Gridley's reticulum stain (×400).

blood agar plates following incubation. However, isolations of a PPLO were obtained from all three birds on the 1396P media and none was obtained from the control birds. .

The poults inoculated with the NTF isolate developed a green diarrhea which began on the 8th day postinoculation. One of these poults that died on that day was found to have

an enlarged liver, a normal sized spleen, and an airsacculitis in one of the anterior thoracic airsacs. There was no evidence of excess exudate in any of the joints of this bird. On the 15th day postinoculation, another poult was autopsied. The liver of this bird was green in color but not too enlarged. The spleen appeared smaller than normal. There was some excess exudate present in both hock joints and the wing joint of the humerus, radius, and ulna of one wing. The remaining three poults were bled and necropsied at the end of the 3-week holding period. There were no liver or spleen lesions in any of these birds. However, each one had an excess of typical, mucoid exudate in the hock and the stifle joints. Two of these poults also had a slight airsacculitis with no other gross lesions of the respiratory tract noticed. Three of the five poults in this group had the airsacculitis lesions. In addition, each had some of the lesions of infectious synovitis.

The histological sections of the liver of the first poult that was killed contained the typical focal accumulations of reticular cells in the liver. The liver sections of the two birds killed at the end of the trial still had the perivascular and sinusoidal reaction to a slight degree, but also had many heterophils throughout the section. Possibly this heterophilic accumulation was due to the airsacculitis that was present in these two birds.

Cultures were taken from the bird killed on the 15th day and from two of the birds killed at the end of the trial. Similar samples were obtained from the same number of birds in the control group. The same cultural procedure with synovial fluid was followed here that had been used for the chicks in this trial. The results of these cultures were the same as for the chicks. No isolations were obtained from the blood agar cultures. However, isolations of PPLO were obtained from all of the poults cultured.

The chicks and poults that had been inoculated with the 1853, Texas, or 298 isolates as well as the controls did not demonstrate any symptoms or lesions throughout the three week postinoculation holding period. However, following one egg passage, these isolates produced swollen hock joints which

contained excess synovial fluid in chicks following intravenous inoculation and reisolations of PPLO were made from these affected birds. None of the poults or chicks in any of these groups had sera positive to the S_6 type PPLO plate agglutination test either before or after the experiment.

Chicken embryos was inoculated with 0.3 ml of a dilution (either 10^{-1}, 10^{-3}, or 10^{-5}) of the same broth culture via the yolk sac route that was injected into the chicks and poults (figure 5). After inoculation, the eggs were incubated in the usual manner and candled every 24 hours. All deaths that occurred within the first 48 hours postinoculation were attributed to inoculation injury and were not included in the results of the trial (table 1).

TABLE I

Mortality of chick embryos inoculated with IS strains

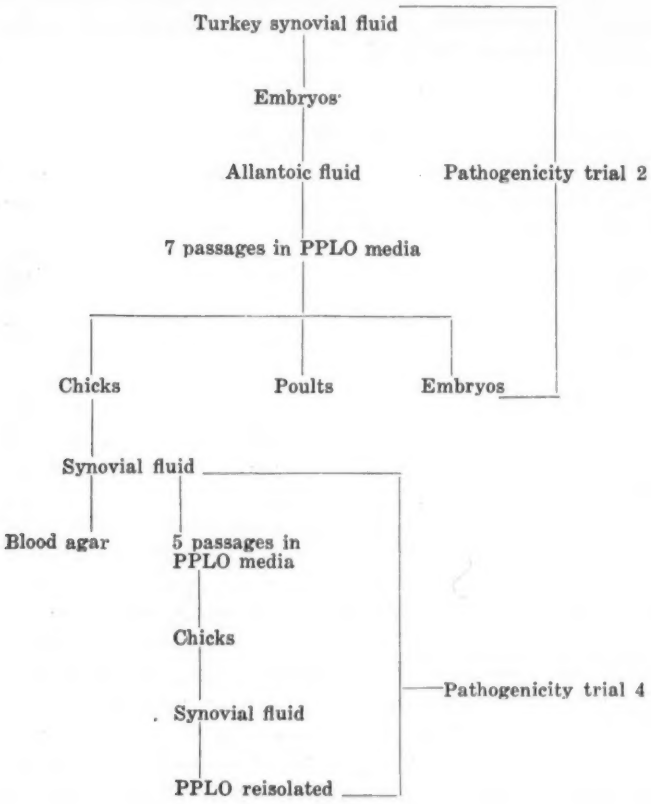
Isolate	Inoculum dilution	Mortality
Uninoculated controls		0/6*
Broth controls		0/6
1853	10^{-1}	0/6
	10^{-3}	1/4
	10^{-5}	3/5
Texas	10^{-1}	2/5
	10^{-3}	4/5
	10^{-5}	5/6
298	10^{-1}	0/5
	10^{-3}	1/6
	10^{-5}	3/6
NTF	10^{-1}	2/6
	10^{-3}	5/5
	10^{-5}	4/5

* Number deaths/Number in group.

All of the embryo deaths occurred between the 4th and the 10th day postinoculation. The incubation period was terminated at this time to facilitate the culturing of the embryos. The yolk and allantoic fluid from two eggs of the 10^{-3} dilution for each isolate were cultured in 1565B media as was described earlier. Isolations of the PPLO were obtained from all groups but the controls. There were some deaths of the embryos inoculated with each isolate. However, the Texas

and NTF isolates appeared to be the most pathogenic for embryos at this time. The exact reason for the higher mortality of the embryos that received the more dilute inoculums is not known. Possibly the thallium acetate in the media affected the embryo at the 10^{-1} dilution enough to alter its ability to support the growth of this agent.

3. The next pathogenicity trial was essentially a continuation of the second trial. The outline below gives the plan of both of these trials and indicates their relationship to each other. The synovial fluid obtained from the NTF and control



groups of chicks in trial 2 was passed serially five times in 1565B media. Subcultures of the second and fifth passages were made to 1396P plates to determine the amount of growth present. Colonies too numerous to count were obtained from both broth passages of the NTF isolate. No growth was obtained from the control broths. The fifth broth passage of these synovial fluid cultures was inoculated into another group of 5-week-old chicks. The broth cultures were diluted with an equal volume of sterile nutrient broth and each chick received one ml of the appropriate diluted culture intravenously.

On the 5th day postinoculation, the birds inoculated with the NTF culture were noted to have pale combs and a greenish discoloration of their droppings. On the 9th day postinoculation, two of the birds in each group were necropsied. One of the birds in the NTF group had a slightly swollen, dark liver and a severely swollen spleen. There was also typical mucoid exudate present in the foot pads and the hocks of this bird. The other bird had a slightly swollen, dark liver but the spleen appeared normal. There was a little gray mucoid exudate present in the hock but none was observed in the foot pads. There were no lesions observed in the control birds necropsied at this time. The remainder of the birds in both groups were necropsied at the end of the 3 weeks holding period. All of the four birds in the NTF group were quite emaciated at this time and had severely swollen foot pads and hocks. These swollen areas were filled with typical mucoid exudate containing some yellow caseous masses.

There were no liver or spleen lesions noted in any of these birds. No signs of lesions were noted in the birds of the control group.

Histological sections of the two birds killed from each group on the 9th day postinoculation were examined. The livers of the NTF birds had the characteristic focal accumulation of reticuloyctes whereas the livers of the controls appeared normal.

Synovial fluid was harvested with a sterile cotton swab and inoculated into a tube of 1308B. Both birds from the NTF

and the control groups that were killed on the 9th day post-inoculation were cultured in this manner. The second broth passages of these cultures were transferred to 1396P plates. Growth of over 100 colonies was obtained from each of the NTF birds cultured. No growth was obtained from the control cultures.

The NTF isolate of PPLO, in its effect on chickens and turkeys, paralleled the signs, gross and microscopic lesions attributed to previously described IS agents.

TABLE II

Effects of intravenous inoculation of a broth culture of the NTF strain of PPLO. Each 4-week-old chick or poult received 1 ml of a one to five dilution of a 72 hour broth culture

Bird no.	Inoculum	Day post-inoculation	Enlarged hock & foot pad	Swollen liver	Swollen spleen	Mucoid exudate present in joints	Airsac-culitis present
Chicks							
1	NTF	9	+*	+**	+	+	0
2	NTF	21	+	+	0	+	0
3	NTF	21	+	+	0	+	0
4	NTF	21	+	+	0	+	0
5	NTF	21	+	+	0	+	0
Poults							
1	NTF	8	0	+	0	0	+
2	NTF	15	+	0	0	+	0
3	NTF	21	+	0	0	+	0
4	NTF	21	+	0	0	+	+
5	NTF	21	+	0	0	+	+
Chicks							
1	Control	9	0	0	0	0	0
2	broth	21	0	0	0	0	0
3	broth	21	0	0	0	0	0
4	broth	21	0	0	0	0	0
5	broth	21	0	0	0	0	0
Poults							
1	Control	15	0	0	0	0	+
2	broth	21	0	0	0	0	+
3	broth	21	0	0	0	0	+
4	broth	21	0	0	0	0	0
5	broth	21	0	0	0	0	0

* Isolations of PPLO obtained from these birds.
**Histological sections were taken from these birds and typical lesions of IS were noted.
+ = lesion was present.
0 = lesion absent.

S_6 type PPLO isolations from field cases of synovitis. Accessions 59478 (isolate CSO), 59592 isolate CSO_1), and 59674 (isolate CSO_2). Three cases involving swollen joints with typical exudate in the hock, and foot pad were investigated. Two of these cases had no gross lesions of the respiratory tract and no history of respiratory signs. One of these cases without any respiratory signs had an enlarged liver and spleen in the bird cultured. Typical S_6 PPLO colonies were isolated in media (swine infusion broth) and in one case after the first passage in embryos. One of these isolates produced joint lesions in chicks following intravenous inoculation. The other two isolates did not have any effect on chicks. It should be noted that at the time these cases were investigated, the DPN media had not been developed. It is possible, therefore that the true ISD type of PPLO might also have been present.

DISCUSSION

Only a very limited study was made of some of the possible variations in the DPN media which appeared to be satisfactory. The 1308B media which contained the basal broth + 0.1 percent DPN + 0.1 percent cysteine was probably the best liquid media. Similarly, the 1396P media which contained the basal agar + 0.1 percent DPN appeared to be the most satisfactory solid media. However, these judgments were derived from observations made from separate trials. Furthermore, a few unexplained failures have occurred with these media which indicate that a more thorough study is needed to clarify the exact requirements of these organisms.

Additional work is also needed to improve the actual cultivation technique. Such manipulations as pipetting the DPN into each tube of broth just prior to its inoculation and the heat inactivation of the swine serum might not be necessary. These possibilities were not investigated in this study. However, the main objective of any study on the cultivation techniques should be to obtain the maximum amount of growth no matter how difficult the required techniques.

The great superiority of the allantoic fluid over the yolk

of the same egg as an inoculum for the isolation of these ISD PPLO in the DPN media was quite surprising. Evidently some of the constituents of the yolk either destroyed or at least made some of the necessary components of this media unavailable for the PPLO. Here again, the cultivation technique becomes more complicated, especially when one is attempting to isolate this agent from chicks or embryos at hatching time. Additional work of this type is needed to compare also such inoculums from affected birds as liver-spleen suspension, blood, sera, etc. which have previously been reported by Benton and Cover[3] to contain this agent.

The fact that these new PPLO which were isolated were serologically distinct from the S_6 type PPLO was not surprising. Snoeyenbos and Olesiuk[15] and Sevoian *et al.*[14] have reported that the agent of infectious synovitis did not induce the production of antibodies to the S_6 type PPLO as judged by the plate agglutination test in birds affected with this disease. This serological characteristic can be used to differentiate them from the S_6 type PPLO. Since no effort was made in this study to determine how readily an S_6 type PPLO will adapt and grow in the media containing DPN and since both types of PPLO have been isolated from cases of synovitis, the serological typing of the organisms becomes the major criterion for determining which of these types of PPLO have been isolated. As indicated before, a study of many cases of synovitis is needed to determine the relative incidence of these two types of PPLO.

The pathogenicity of these new PPLO resembled very closely that described for the agent of infectious synovitis. The IS agent has been known by all who have worked with it to lose its pathogenicity for birds easily whenever it is not maintained by continual or at least periodical bird passages. It was not surprising, therefore, that in our second pathogenicity trial, the NTF isolate was the only isolate still pathogenic for birds. The other isolates had all been through several egg passages prior to this trial whereas the NTF inoculum represented the first egg passage of the original synovial fluid. The additonal seven passages in artificial media probably were

enough to cause all the isolates but the NTF to lose their pathogenicity for birds. It is interesting to note, therefore, that these new PPLO also appear to differ from the S_6 type PPLO in pathogenicity.

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SUMMARY

1. This study was conducted to isolate and identify the etiological agent of infectious synovitis (IS). Lecce's[10] isolation of a PPLO was confirmed when a PPLO was isolated by his method not only from his isolate of IS, but also from three other IS isolates procured from other laboratories.

2. After many attempts, a satisfactory culture medium was developed which eliminated the *Staphylococcus aureus* streak on plates as required by Lecce's technique. The solid media was PPLO agar (Difco) which contained 0.1 percent diphosphopyridine nucleotide (DPN), 10 percent heat inactivated swine serum, 1 to 2,000 concentration of thallium acetate, and 1,000 units of penicillin per ml. This medium was incubated in the candle jar. The broth media was PPLO broth without crystal violet (Difco) which contained 0.1 percent DPN, 0.1 percent L cysteine (free base), 10 percent heat inactivated swine serum, 1 to 1,000 concentration of thallium acetate per ml. and 1,000 units of penicillin per ml. The broth media was incubated aerobically.

3. With these media, colonies similar to those obtained by Lecce's technique were isolated from infectious allantoic fluid or synovial fluid of seven different isolates of the IS agent. Two of these isolates were obtained from field cases of IS at this laboratory. The colonies obtained on this media

were typical PPLO colonies, similar to an S_6 type PPLO in morphology. The organisms grown from each of these seven isolates of the IS agent fermented glucose and maltose, but not sucrose, lactose, or mannite. All seven isolates were found to be serologically distinct from S_6 type PPLO by the plate inhibition test of Edward and Fitzgerald.[6]

4. Infectious allantoic fluid and yolk from the same egg were compared as inoculums for broth media containing DPN in two different trials. Each trial contained inoculums which represented at least five of the different isolates as well as control groups. In both trials the allantoic fluid was far superior to the yolk as an inoculum for the growth of these organisms in this media.

5. Cultures derived from three isolates were found to be pathogenic for chicks in one trial. An isolate recently obtained from a turkey was still pathogenic for chicks and turkeys after seven passages on artificial media. The culture reisolated from these experimental chicks and serially passed five times in artificial media, was still pathogenic for chicks following intravenous inoculation. The disease in chicks and turkeys produced by inoculation with these new PPLO paralleled the signs, gross and microscopic lesions attributed to IS agents previously described.

6. Three isolates of S_6 type PPLO were obtained from synovial fluid of field cases which appeared to be cases of IS. One of these isolates was found to be pathogenic and produced swollen foot pads, shanks, and hocks in chicks following intravenous inoculation. Since these cases were cultured before the media containing DPN was developed, both the S_6 and the new type of PPLO described in this paper may have been present.

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BOOK REVIEWS

Methods for the Examination of Poultry Biologics. By National Academy of Sciences National Research Council, Washington, D. C. Publication tion 705, 1959. 127 pp. Price $3.00.

This publication is a manual of standard procedures and methods used for investigating poultry diseases and testing vaccines for poultry. It should prove valuable as a guide for those who are beginners and inexperienced in the avian disease field and as a reference for those engaged in poultry disease research and diagnosis and vaccine production.

Although the material presented is primarily for those engaged in evaluating poultry vaccines, the information has much broader usefulness in research and diagnosis. The reader is introduced to the manual by way of a brief explanation of housing, facilities, and husbandry practices for the care of laboratory animals. Biologic definitions are spelled out for better communication among workers. Considerable detail is included on laboratory methods some of which have widespread application whereas others apply to the manipulation of specific poultry pathogens. Also presented are standard quantitative methods which are used to determine the identity, purity and potency of bacterial or viral cultures and vaccines. The explanation on the use of the chicken embryo for virus isolation, modification and propagation and for serum and virus neutralization tests are good. However, the application of tissue culture techniques for similar uses was not included.

It was especially noteworthy that the authors emphasized the importance of experimental design. Some aspects and errors of biological and statistical samplings are presented. This is an area which needs considerable elaboration and application.

Because of the nature of the subject matter covered by this manual, it is important that it be revised periodically if it is to remain valuable. By increasing the scope, it is conceivable that it may become the most valuable laboratory manual in the poultry disease field.

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The Avian Embryo: Structural and Functional Development. By Alexis L. Romanoff. New York: The Macmillan Co., 1960. 1305 pp; 426 illustrations. Price $35.00

Professor Romanoff was born and educated in St. Petersburg, Russia, and studied at Tomsk University, Vladivostok Polytechnic Institute, and St. Petersburg Military Engineering Academy. In 1921 he came to the United States, became a citizen in 1927 and received his Ph.D. degree from Cornell University. In 1949 he published a monumental work entitled "The Avian Egg".

The field of embryology has been of interest for centuries, and numerous investigations by a host of workers in many countries, have left us a rich heritage. The progress made in our understanding of the avian embryo has advanced on many fronts as the techniques and tools of the morphologist and physiologist evolved. We are now on the threshold of new advances in our knowledge of the biological, chemical and physical aspects of development and the avian embryo will play an ever increasing role in research. A reminder of the importance of specific developmental documentation and its application, was published in 1952 by the New York Academy of Science in a monograph entitled "The Chick Embryo in Biological Research".

Any attempt to summarize and synthesize the scattered world literature concerning the embryogeny of birds, and incorporate it in one volume is a prodigious task. An adequate historical introduction would have to include the embryological data of Hippocratic writings, through Aristotle and Galen, to the revival of interest in the 14th, 15th and 16th centuries (Needham, 1934 A History of Embrology). Dr. Romanoff has accomplished much in his study of more than 7,000 original papers, of which two-thirds were in languages other than English. Included in the text in addition to original observations are over 2,700 literature citations with page references which provide valuable assistance in one's search for source materials. An omitted reference source which should be mentioned is "A Bibliography of Birds" by Reuben Strong, published by the Chicago Museum of Natural History as Vol. 25 parts 1, 2, 3, and 4 (1939-1959). Obviously one cannot include

in one volume or even peruse in one's lifetime, all of the published anatomico-physiological facts of avian development. However the author and his assistants have made a masterful attempt to review and assess our recorded knowledge of the form and function of the developing bird. Over 100 species of birds are referred to in the text and cited in the index. The text is supplemented by many useful illustrations redrawn from the original papers, although in some cases these have suffered from excessive reduction.

The completeness of the subject matter coverage is most gratifying and although omissions can be cited, one working in the field takes such shortcomings for granted. The arrangement of the Table of Contents and Index, facilitates rapid reference and provides a handy assessment of the depth of coverage. It is unfortunate that the price is so high since it is such a desirable reference text to own.

HOWARD E. EVANS

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ABSTRACTS OF PART OF THE PAPERS PRESENTED AT THE 32nd NORTHEASTERN CONFERENCE ON AVIAN DISEASES, JUNE 27-29, 1960, UNIVERSITY OF MAINE, ORONO, MAINE

The papers presented here in abstract represent part of those given at the meeting. The remainder of the papers are presented in their entirety elsewhere in this issue.

ACTION BY POULTRY INSPECTION TO HELP REDUCE CONDEMNATION LOSSES

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During 1959 nearly 5 billion pounds of poultry was certified as wholesome while about 90 million pounds of poultry was condemne as unwholesome. The Poultry Inspection Service of the U. S. Department of Agriculture has long recognized the seriousness of the economic impact on the industry resulting from condemnations at official establishments. To enable industry to understand condemnation losses in their proper perspective, the Department is drawing attention to the fact that 10 to 20 percent of condemnations often are caused by faulty processing. The processor can do the most good when the condemnations are due to contamination, misbleeds (cadavers) and bruises. The latter item, bruises, results in serious loss from trim-out and down-grading of finished products, in addition to the total carcass condemnation for this

condition. Live poultry handling in all stages may contribute to this loss.

Condemnations due to disease processes are for the most part made on the basis of determining the nature, extent, and duration of the disease, or combination of disease processes, rather than upon specific diagnoses. Exceptions to this are cases of tuberculosis and leucosis in its several forms. Those two conditions are manifested by readily identifiable gross lesions and can be reliably diagnosed at post-mortem inspection. The other condemnations due to disease are grouped under general terms such as septicaemia-toxemia, inflammatory process, tumors and the like. Systemic disturbances under these headings are determined by observing loss of tissue tone, color sheen and texture. These are reliable evidences of metabolic imbalance and are taken into consideration with any specific pathological lesions present.

Our official report forms show the disease-caused losses in the following order by volume: In fryers and broilers, septicaemia-toxemia, first; inflammatory processes, second; leucosis, third; and tumors, fourth. Bruises outrank tumors as cause for losses; the numbers lost due to contamination, overscalds, and misbleeds are greater than the combined losses due to leucosis and tumors. While losses under the heading of septicaemia-toxemia rank first in volume on our reports, condemnations due to respiratory disorders (principally C.R.D. and Newcastle disease) are responsible for more condemnations than any other single disease syndrome in classes of young chickens. In fowl (adult chickens) septicaemia-toxemia ranks first; inflammatory processes, second; tuberculosis, third; leucosis, fourth; and multiple tumors, fifth. Here again processing-caused losses are nearly as serious as those for broilers.

The American Association of Avian Pathologists has, through the efforts of its president, set up a committee to promote cooperation between inspection personnel and diagnostic laboratories. We believe this concerted effort by so many mutually interested groups should, if carried out as team work, result in much benefit to the industry and the conumer. The Poultry Inspection Branch of the Agricultural Marketing Service stands ready to do everything humanly possible to be helpful in these efforts within the limits of the authority provided in the Act and the Regulations.

NEWCASTLE DISEASE: PRELIMINARY FIELD TESTS OF *BETA*-PROPIOLACTONE-KILLED VACCINE

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Research workers in the United States believe that continued use of live-virus vaccines against Newcastle disease precludes eradication of the disease. In many cases, these agents precipitate outbreaks of other respiratory infections such as infectious bronchitis and chronic respiratory disease (air-sac infection).

In our investigations on Newcastle disease, we have developed a safe, killed-virus vaccine that is similar in potency to the live-virus vaccines. From our experience and field study conducted in four United States areas (Northeast, North-central, Southwest, and the Delaware-Maryland-Eastern Shore) and in Nigeria, Africa, we believe that a *beta*-propiolactone-killed vaccine gives adequate protection in both brioler and layer flocks. The immunity induced by this vaccine has been particularly favorable in field tests. Many former proponents of live-virus vaccines may now re-evaluate the efficiency of killed-virus vaccine.

We believe that the general use of a good killed-virus vaccine will offset production and administration costs, since such use will improve the quality and quantity of birds for domestic and international markets. Unfortunately, a *beta*-propiolactone-killed vaccine is not yet available commercially. We hope it will be available soon for poultry producers.

EFFECT OF FURAZOLIDONE MEDICATIONS UPON THE INFECTIVITY OF *S. PULLORUM* IN EXPERIMENTALLY INOCULATED CHICKENS

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One trial was conducted using 6-month-old White Rocks and two trials with Rhode Island Red hens, 14 and 19 months of age. Birds were inoculated either intratracheally or intramuscularly with a broth culture of *S. pullorum*. Furazolidone was administered by tablets and through medicated feed (0.022%) beginning three weeks after initial inoculation. Medication continued for three weeks in trials 1 and 2, and for four weeks in trial 3. Part of the birds were necropsied at end of medication period and the remainder several weeks later.

In the first trial, nine of the 10 treated birds were serologically positive with a group GMT of 279 at the end of medication period, and *S. pullorum* was recovered from three birds. All 10 of the nonmedicated birds were serologically positive with a GMT of 496 and seven birds yielded the organism.

In the second trial, 11 of the 15 medicated birds had positive agglutinin titers with a group GMT of 64 and the pullorum organism was recovered from eight birds at the end of medication. All 13 of the nonmedicated birds were serologically positive with a GMT of 188 and 12 birds yielded *S. pullorum*. The remaining half of the birds were examined 3 weeks after removing medicated feed. The results were similar to those observed in the first group.

In the third trial, 146 birds were inoculated. At the end of medication, all 25 of the medicated and 24 of the nonmedicated birds were serologically positive with GMT's of 236 and 375, respectively. *S. pullorum* was recovered from one medicated and six nonmedicated birds. Remaining birds were examined 4 and 5 weeks later. Results were similar to those observed in the first group of this trial. Thirty uninoculated birds in cohabitation with the inoculated birds in each pen from beginning of medication to the termination of the experiment remained serologically and bacteriologically negative.

TISSUE CULTURE PROPAGATION AND PATHOLOGY OF A CELO VIRUS

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An agent causing a cytopathogenic effect, differing from that caused by infectious laryngotracheitis (ILT) virus in chicken-embryo-kidney cell cultures, was recognized in the 17th serial passage of an ILT virus. The agent affected individual cells causing the nucleus to enlarge several times normal size and the nuclear contents to appear stippled. Plaques were produced in inoculated sheets of kidney cells overlaid with agar.

One ml of cell culture fluid contained 10^9 plaque forming units (PFU) and immune serum neutralized up to 10^6 PFU.

On subsequent passages in the chorioallantoic sac, this agent killed chicken embryos at 10^{-10} when 0.2 ml was inoculated per embryo. Immune serum neutralized up to 10^6 ELD_{50}. The agent was passed serially on chorioallantoic membrane (CAM) and produced lesions similar to ILT virus. Histopathologic lesions in the CAM were also similar to ILT but no characteristic intranuclear inclusion bodies were observed.

Birds inoculated into the infraorbital sinus and trachea with cell culture material showed only localized histologic lesions in the sinus. These birds produced a rise of 10^5 VND_{50} in serum antibody level 4 weeks post inoculation.

Serology and comparative histopathology identified the agent as a CELO virus.

Supported in part by funds provided by the Animal Disease and Parasite Research Division, A.R.S., U.S.D.A.

ISOLATION AND SEROLOGIC TYPING OF AVIAN PLEUROPNEUMONIA-LIKE ORGANISMS (PPLO)* M. E. Tourtellotte, J. G. Aftosmis, E. Bacon, and R. E. Luginbuhl, *Department of Animal Diseases, University of Connecticut, Storrs, Connecticut.*

A simple cultural method for the isolation of avian PPLO is described which makes possible the testing of large numbers of birds with a minimum of laboratory manipulation.

Recovery of pathogenic PPLO from artificially inoculated birds was 90 to 100 percent after 21 days, dropping to 80 percent at 30 days. Results in the field were not as good. The value of this isolation technique in vaccination trials and in testing the efficacy of chemotherapeutic agents against PPLO will be discussed.

The selective isolation of pathogenic PPLO in the presence of nonpathogens and the rapid typing of fresh isolates by addition of hyperimmune rabbit serum to media (Edward, D. G., The pleuropneumonia group of organisms: A review, together with some new observations. Jour. Gen. Microbiol., *10*, 27-64, 1954.) will also be discussed.

Serologic testing of a number of PPLO isolates showed the existence of several serotypes. Results of cross-agglutination and agglutinin-adsorption tests revealed a complex antigenic make-up of some nonpathogenic strains; e.g., strain A cross reacted with strain B; however, adsorption of antiserum B with antigen A did not remove agglutinins for antigen B.

All pathogenic PPLO tested, however, were found to be serologically homologous.

* Supported in part by funds from the U.S.D.A., Agricultural Research Service.

THE *IN VIVO* AND *IN VITRO* ISOLATION OF AVIAN ENCEPHALOMYELITIS VIRUS (AEV)*. J. Hwang, R. E. Luginbuhl and C. F. Helmboldt. *Department of Animal Diseases, University of Connecticut, Storrs, Connecticut.*

AEV isolations by intracerebral inoculation of chicks and by chick kidney cell culture passages were carried out in parallel with pooled brain samples from 30 individual field cases diagnosed by histopathology. Sixteen cases or 53.3% were positive with an average incubation period of 15.5 days by the *in vitro* method while 14 cases or 46.7% were positive with an average incubation period of 22.5 days by the *in vivo* method.

The titers of the 16 virus strains isolated by chick kidney cell cul-

ture passages were $10^{-7.6}$ to $10^{-9.5}$ / 0.1 ml in the cell culture system and the hyperimmune serum neutralized $10^{7.1}$ to $10^{8.8}$ cell culture infective doses.

Addendum—Laboratory studies made since the presentation of the above data indicate that the isolations made in tissue culture were not AEV but were related to a prototype CELO virus. The AEV used to prepare the hyperimmune serum apparently contained CELO virus as well as AEV. The CELO virus was capable of producing ataxia and histologic lesions in chicks which might be confused with those produced by AEV.

* Supported in part by funds from the American Poultry and Hatchery Federation.

EVALUATION OF CURRENT CONTROL MEASURES RECOMMENDED FOR THE INTESTINAL HAIRWORM (*CAPILLARIA OBSIGNATA*) OF POULTRY

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Twelve pens of 71-week-old sex-link hens parasitized with capillaria and cecal worms were divided into six groups of two pens each. Group 1 received Hygromycin B* for 30 days continuously in the feed. Group 2 received additional Vitamin A for 10 days in the feed. Group 3 received 3 days of Milibis** in the feed the 25th through the 27th day of the test period. Group 4 received a triple dose of piperazine in the water the 20th day followed by 10 days of additional Vitamin A in the feed. Group 5 received a triple dose of piperazine in the water the 25th day. Group 6 received regular feed and was maintained as untreated controls. Weekly egg counts, with a modified Stoll technique, were discontinued because of the erratic results. Worm counts at the termination of the 30 day test period were made using Frazier's technique. Group 1 (Hygromycin treated) showed a significant decrease in the number of adult capillaria at necropsy over the untreated control group. There was no significant decrease in the number of capillaria in the other test groups.

Egg production at the beginning of the experiment was approximately 65 percent. At the end of the 30 day test period Group 1, Hygromycin treated birds, had less than 5 percent production. Group 4, piperazine plus Vitamin A, was under 15 percent production. Production in other groups had dropped gradually to 50 percent.

Hygromycin was the only agent used which significantly reduced the number of capillaria. However, it caused a marked drop in production as did piperazine plus Vitamin A.

*Eli Lilly and Company, Indianapolis, Indiana
**Sterling-Drug Inc., Rensselaer, New York

NON-SPECIFIC PLATE AGGLUTINATION REACTIONS WITH PPLO ANTIGEN

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A large number of positive reactions to the PPLO serum-plate agglutination test were observed in a commerical flock of breeder turkeys that had been vaccinated with erysipelas bacterin (Duovax-Cyanamid) within the previous eight weeks. Non-vaccinated turkeys from the same flock were negative to the PPLO agglutination test.

Under experimental conditions a group of turkeys and chickens at the laboratory were vaccinated with erysipelas bacterin (Duovax-Cyanamid) subcutaneously. The vaccinated birds as well as comparable unvaccinated controls were bled at weekly intervals. Positive plate agglutination reactions with PPLO antigen (ASL) appeared by the eighth day and almost completely disappeared by the 29th day post-vaccination. A few weak reactions persisted until the 36th day in the turkeys. No reactions occurred in the controls.

A similar vaccination trial with cholera bacterin (Glandolac Company) in turkeys did not produce PPLO plate reactions.

The lot of antigen used in these experiments had previously been shown to be agglutinated by serums from PPLO (S_6) inoculated chickens and turkeys.

REPORT ON THE PULLORUM DISEASE TESTING RESULTS FOR THE 32nd NORTHEASTERN CONFERENCE ON AVIAN DISEASES

A summary of pullorum testing data was collected and compiled for 1959-60 by a special committee of the Conference (W. R. Teeter, Chairman, F. W. Rea, and H. Van Roekel). Fourteen states and two Canadian provinces cooperated in furnishing the testing data. The testing results concerning chickens and turkeys are listed in the table below.

In addition to the chickens and turkeys tested, the following fowl other than chickens were tested: 162 bantams, 3,502 ducks, 605 game birds, 71 geese, 16 grouse, 1,207 guineas, 1,013 partridges, 23 peacocks, 387 pea fowl, 25,916 pheasants, 74 pigeons, 1,564 quail, 44 wild turkeys, and 4 wood ducks. In only one instance was pullorum disease detected in fowl (pheasant) other than chickens.

Special credit should be extended to Dr. Teeter for summarizing the data submitted. The following is a condensed summary of the testing data by states and provinces.

Submitted by:
H. Van Roekel, Permanent Secretary

Summary of Pullorum Disease Testing Data Reported by Northeastern Conference on Avian Diseases

	1959-60						
	1	2	3	4	5	6	7
Conn.	118*	628,508	0.006	115	0	117	628,501
	24**	26,951	0.007	24	1	24	26,951
Del.	277	537,714	0.009	273	4	272	532,482
	6	1,102	0.00	6	0	6	1,102
Maine	247	1,734,746	0.00	245	0	244	1,731,513
	1	567	0.00	1	0	1	567
Md.	454	258,474	0.019	431	3	451	257,169
	14	6,857	0.00	14	0	14	6,857
Mass.	160	690,537	0.001	158	0	94	503,236
	18	15,944	0.00	18	0	12	10,780
N.C.	1,863	2,731,362	0.003	1,841	9	1,849	2,701,262
	50	76,203	0.03	45	0	47	73,303
N.H.	167	640,042	0.00	157	0	157	615,210
	11	13,664	0.00	11	0	11	13,664
N.J.	181	528,635	0.003	159	5	162	501,001
	12	6,741	0.00	12	0	12	6,741
N.Y.	152	570,663	0.00	134	0	129	522,631
	24	23,520	0.00	24	0	24	23,520
Nova Scotia	28	78,790	0.00	28	0	28	78,790
	7	952	0.00	7	0	7	952
Ontario	2,283	1,513,787	0.05	2,255	0	2,223	1,479,179
	138	109,862	0.00	138	0	138	109,862
Pa.	942	1,568,403	0.02	927	14	926	1,526,840
	68	32,109	0.06	66	0	66	31,347
R.I.	28	34,769	0.00	26	0	26	27,538
	2	581	0.00	2	0	2	581
Vt.	107	207,813	0.00	107	0	107	207,813
	6	4,437	0.00	6	0	6	4,437
Va.	1,079	1,131,408	0.029	961	20	1,068	1,121,929
	255	158,178	0.001	253	0	255	158,178
W. Va.	398	114,762	0.00	398	0	398	114,762
	24	12,318	0.00	24	0	24	12,318

1 = Number of flocks tested.
2 = Number of birds tested.
3 = Percent positive (based on number of birds tested).
4 = Number of flocks 100% tested and with no reactors.
5 = Number of breaks in flocks 100% tested and with no reactors the previous season.
6 = Number of pullorum-clean flocks.
7 = Number of pullorum-clean birds.
* = These figures under each state represent chickens.
** = These figures under each state represent turkeys.

REPORT OF THE COMMITTEE ON NOMENCLATURE AND REPORTING OF DISEASES NORTHEASTERN CONFERENCE ON AVIAN DISEASES JUNE 1960

The purpose of this report is to present information on the incidence of disease in the area encompassed by this Conference. Furthermore it is hoped that a nomenclature and system of reporting of diseases can be developed which may serve to standardize the reports from the various laboratories.

The statistics are based on the calendar year 1959 with the exception of those for Canada and Delaware which are based on a fiscal year. Each unit statistic represents a single case or consignment to the laboratory and therefore approximates an outbreak of disease on a premises. The figures are reported by states rather than by member laboratory. Statistics shown for Canada were compiled from those submitted by the Ontario Veterinary College, Guelph and the Regional Laboratories at Ridgetown and Kemptville.

The incidence of disease is reported for age groups for chickens which coincide roughly with the brooding, growing and adult life of the birds. A separate category indicates incidence of disease in species other than chickens. Whenever possible the identity of the species is indicated: T-turkey; Ph-pheasant; D-duck; Pkt-parakeet; Pgn-pigeon; Qu-quail; Gu-guinea; WD-wild duck; Sw-swan; Ter-tern; Dov-dove; Can-canary. More than one species were combined and designated by letter O.

All infectious and contagious, parasitic and nutritional diseases plus such miscellaneous and unclassified conditions and diseases as may be of significance or interest are reported. However limitations of space and variations in systems of reporting by the various laboratories make it necessary to be somewhat arbitrary in preparing the report and some discrepancies and omissions are unavoidable.

Specific nomenclature is used wherever possible to indicate specific disease entities. However for expediency some classifications are used in a generic sense and singleness or identity of etiology is not indicated. Ascaridiasis, coccidiosis and pox are examples. Nutritional diseases are classified according to clinical syndrome. Thus under the term rickets are included cases of improper calcification of bones whether due to avitaminosis D, a deficiency or imbalance of minerals as calcium or phosphorus, or an excess of sulfur in the diet. The statistics are presumed to be essentially accurate even though not all diagnoses are based upon the isolation or identification of the etiological agent.

Certain unavoidable discrepancies are indicated by letter which also appears by the statistic to which it applies.

a - figure includes all chickens regardless of age
b - figure includes all avian species
c - figure includes all worms
d - figure includes all forms of Leukosis

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Summary of Poultry Diseases
Reported by Northeastern Conference on Avian Diseases
1959

State		Conn.	Del.	Maine	Md.	Mass.	N.H.	N.J.	N.Y.	N.C.	Penn.	R.I.	Vt.	Va.	W.Va.	Canada
Consignment Totals		1544	3541	1197	1881	1555	1915	6313	5772	4833	5621	1002	394	4947	482	4323
Acariasis	1-5 Mos			1		1		b 56	3		a 41		a 4			14
	+ 5 Mos				2	12	8		72			3				31
	Other								0-1							0-1
Anatipestifer infection	Other								0-111							
Aneurysm, dissecting	Other					T-3	T-2		T-3	T-2	T-10					T-16
Ascaridiasis	0-4 Wks	a 41	4		4			b983	5	22	a534		a16	11		
	1-5 Mos		54	12	64	5	11		59	375		1		118	c 9	66
	+ 5 Mos		5	62	26	21	34		299	203		6		172	c11	243
	Other		T-4		0-2	T-1			0-3	T-12	0-16				cT-1	0-4
Aspergillosis	0-4 Wks		55	2	46	1		b 12	9	24	a 69			28	4	7
	1-5 Mos		14	2	2		1		19	33				5	1	11
	+ 5 Mos					3			11	22				3		16
	Other		T-9	0-2	0-11	0-4			0-43	0-11	0-48			T-28	T-2	0-69
Avitaminosis A	0-4 Wks		1		5			b 31		1	a 8			2		3
	1-5 Mos				13				3	4				2		4
	+ 5 Mos								7	2						6
	Other				Ph-1					T-4	0-2			T-1		
Blue Comb Complex	0-4 Wks	a 10						b143		2	a129		a 6	1		
	1-5 Mos		18		8				2	11				23	5	3
	+ 5 Mos		6		10	1	8		28	17		1		99	7	28
	Other		T-4		0-2		T-1		0-10	T-30	0-43			T-495	0-12	T-2
Botulism (Limberneck)	1-5 Mos	a 1	2		13	1	1	b 32	16	17	a 8			4		
	+ 5 Mos				4				2	3				2	1	
	Other		D-2		0-8				0-7	0-15	0-9			T-7	T-1	
Bronchitis, Infectious	0-4 Wks	a 15	33		8	2	2	a 46	13	8	a102		a 1	1		9
	1-5 Mos		60	7	34	23	22		17	47		10				20
	+ 5 Mos		1	2	13	15	1		38	17		3			2	15
Capillariasis	1-5 Mos	a 129	1	15	2	5	13	b778	11	45	a273		a17	2		40
	+ 5 Mos			95	8	76	139		159	93		14		5		167
	Other				0-2	0-3			0-4	T-2	0-16		T-1			0-9

State		Conn.	Del.	Maine	Md.	Mass.	N.H.	N.J.	N.Y.	N.C.	Penn.	R.I.	Vt.	Va.	W.Va.	Canada
Consignment Totals		1544	3541	1197	1881	1555	1915	6313	5772	4833	5621	1002	394	4947	482	4323
Cholera, fowl	1-5 Mos		10			1		b 113	3	37	a 82		a15	2	1	2
	+ 5 Mos			8	16	23	4		47	59				14	3	23
	Other	T-3	D-1		O-4	O-5	T-1		O-139	T-10	O-17		T-1	T-51	T-12	T-20
Chronic Respiratory Disease & Infectious Sinusitis	0-4 Wks	a 202	136	18	27	3	18	b 509	26	41	a 626	2	a23	54	30	48
	1-5 Mos		775	231	134	166	90		435	550		24		284	71	314
	+ 5 Mos		12	25	37	80	19		216	47		17		65	18	194
	Other		O-37		O-28	O-18			O-11	O-19	T-46			T-236	O-16	O-122
Coccidiosis	0-4 Wks	a 107	53	11	40	11	2	b1697	39	90	a 483	1	a28	75	1	39
	1-5 Mos		347	91	146	96	62		502	544		24		239	36	343
	+ 5 Mos		6	36	28	60	23		192	132		5		41	2	219
	Other	O-2	T-10		O-13	T-30	T-1		O-32	O-54	O-60	Pgn-1	O-4	O-201	O-23	O-106
Coryza, Infectious	0-4 Wks	a 6	1		4		1	b 10		6	a 9		a 1	2		1
	1-5 Mos		1		9					22				2		3
	+ 5 Mos		1		7	8	1		3	14				5		6
Encephalomalacia	0-4 Wks	a 6		2	9	3			1	33	a 6			2		
	1-5 Mos			1		2	16		4	8						
Encephalomyelitis, Avian	0-4 Wks	a 61		17	33	35	23	b 43	81	41	a 91	6	a10	1	10	65
	1-5 Mos			2	1	1	3		8	20					4	13
	+ 5 Mos					6										
	Other				Ph-2				O-2							
Encephalomyelitis, Eastern (Equine)	Other	Ph-31				Ph-1		O-19	O-12	Ph-3	Ph-1	O-2				
Enteritis, unidentified	0-4 Wks	a 2	44	3	46		1	b 269	1	4	a 195		a 2	33	8	40
	1-5 Mos		306	28	72	1	7		52	67				191	5	118
	+ 5 Mos		11	31	40	4	20		25	30		105		135	7	92
	Other		T-29	O-3	O-7				O-5	O-46	O-17	Pgn-1		T-334	T-7	O-144
Enteritis, Ulcerative	0-4 Wks	a 7						a 74	2		a 3					
	1-5 Mos					8			50							
	+ 5 Mos					1			4							3
	Other	T-1			Qu-1			Qu-4	O-3	Qu-1	O-10			Qu-1		T-3
Erysipelas	+ 5 Mos					1		b 3	3		a 3					1
	Other	T-2	T-8		T-6	T-14	T-1		O-7	T-22	O-20		T-2	T-23		T-32
Hemorrhagic syndrome	0-4 Wks	a 18	2		6		47	b 403		3	a 179		a 3	12		4
	1-5 Mos		91	1	25	6	95		141	33				147	2	63
	+ 5 Mos				2		2		20	6				11	1	3
	Other	T-1	D-1		Ph-1				Ph-1	T-9	T-7			T-11		

State		Conn.	Del.	Maine	Md.	Mass.	N.H.	N.J.	N.Y.	N.C.	Penn.	R.I.	Vt.	Va.	W.Va.	Canada
Consignment Totals		1544	3541	1197	1881	1555	1915	6313	5772	4833	5621	1002	394	4947	482	4323
Hepatitis, Duck Virus	Other								D-99							
Hepatitis, Infectious Avian (Vibrionic)	0-4 Wks	a 68			9			b 122	1	2	a 70		a 8			
	1-5 Mos		1		60	7	1		23	33						8
	+ 5 Mos		3		5	22	8		244	56				1	1	3
	Other									T-1	T-2					T-1
Hepatitis, unidentified	1-5 Mos	a 5		6				b 232	7		a .25				1	1
	+ 5 Mos			10	9	4			33					3	2	21
	Other	0-2	0-2		T-1						0-2				T-1	0-2
Hexamitiasis	1-5 Mos			5						1						
	+ 5 Mos			2						1						
	Other		T-1	T-1	T-1			Qu-2		T-2	0-11					T-1
Histomoniasis	0-4 Wks	a 21			3			b 440	12	10	a 229			1		
	1-5 Mos		8		9	5	12		137	155				19	1	4
	+ 5 Mos				2	7	7		58	72		1		11		5
	Other	0-3	T-14		0-22	0-17	T-4		0-39	T-56	0-40		0-4	0-58	T-9	T-26
Laryngotracheitis, Infectious	0-4 Wks	a 53			4			b 101			a 44		a 2			
	1-5 Mos			9	28	4	1		1	11		7		9		8
	+ 5 Mos			26	2	3	4		8	7		1		6	1	8
Leucocytozoon infection										T-11						
L Erythroblastic)	1-5 Mos	a 4														1
E Myeloblastic)	+ 5 Mos			1	1	2			10		a 18	2				5
U Neural	0-4 Wks	a 39	5			1		bd	1	1	a 314		a 9	2		2
K	1-5 Mos		154	37	19	61	d145	1570	212	163		10		107	d 4	154
O	+ 5 Mos		16	14	1	41	d200		120	37		6		39	d12	71
S	Other		T-2				dT-2			T-2						T-1
I Ocular	0-4 Wks										a 77			2		
S	1-5 Mos		17	2	132				6	6				12		10
	+ 5 Mos		10	5	42	8			72	26				19		46
C Osteopetrosis	1-5 Mos	a 1	1			1			9	4	a 15			2		2
O	+ 5 Mos			2		2			13	5		1		3		2
M	Other									T-1						
P Visceral	0-4 Wks	a108		1	3				2		a 704		a32	6		1
L	1-5 Mos		7	43	5	23			101	206		2		100		93
E	+ 5 Mos		42	103	70	158			503	248		272		116		375
I	Other		T-6			T-6				T-2	T-2					0-9

State		Conn.	Del.	Maine	Md.	Mass.	N.H.	N.J.	N.Y.	N.C.	Penn.	R.I.	Vt.	Va.	W.Va.	Canada
Consignment Totals		1544	3541	1197	1881	1555	1915	6313	5772	4833	5621	1002	394	4947	482	4323
Newcastle Disease	0-4 Wks	a 79	48		69	1	1	b 123	14	19	a 36	3	a 5	6		
	1-5 Mos		80		54	5	1		70	84		13		10	3	
	+ 5 Mos				1	5	2		50	17		2		1	1	
	Other	T-1			Gu-1				T-1	Ph-1	T-1					
Omphalitis	0-4 Wks	a 12	59	29	18	8	59	b 50	52	80	a 55	3	a 7	13	3	21
	1-5 Mos				2				1	21						
	Other	WD-1	T-1	T-2	T-2	T-1	T-3		D-47	0-4	0-10			0-2		0-6
Ornithosis & Psittacosis		Pgn-2				0-2		Dov-1	Can-1		0-3					
Paracolon infection	0-4 Wks				1					1						5
	1-5 Mos				2											
	+ 5 Mos			2						6						2
	Other					T-1					T-1		T-1			0-4
Paratyphoid infection	0-4 Wks	a 4	2		1	3			5	12	a 1			13	1	5
	1-5 Mos								3	10						5
	+ 5 Mos				2	9			4	11				2		2
	Other	Ph-1			Pkt-1	0-20	T-1	0-11	0-26	T-4	0-5			0-7		0-17
Pediculosis	1-5 Mos	a 1	1					b 327	2	5	a 144		a 3	5		2
	+ 5 Mos		1	1	3	26	3		83	1				10	4	16
	Other					Ter-1			0-9		0-8					0-1
Peritonitis	0-4 Wks	a 1		2			1	b 426	1		a 136					2
	1-5 Mos			4			2		11					2		1
	+ 5 Mos		2	11	8	9	1		290					10	10	96
	Other			Sw-1	0-3	T-1			0-48		T-1		T-1	0-3	T-1	T-1
Perosis	0-4 Wks	a 2	1	1				b 15	9	1	a 25		a 1			
	1-5 Mos			3		3	11		30	10				6		18
	+ 5 Mos								5	2						2
	Other			D-1					0-9	Ph-1	0-9		T-1	T-10		0-7
Pox	0-4 Wks	a 3	1					b 89			a 44					
	1-5 Mos		4			4			3	41				3		2
	+ 5 Mos		2		16	35	2		32	31		2		19		28
	Other					0-12			0-8	T-2	0-5			T-16		0-1
Pullorum Disease	0-4 Wks	a 1			1	1		b 2	13	24	a 23			9	2	15
	1-5 Mos								4	7						3
	+ 5 Mos			1	22	9			8	17				1	1	2
	Other	0-2							0-1	T-1				T-2		

State		Conn.	Del.	Maine	Md.	Mass.	N.H.	N.J.	N.Y.	N.C.	Penn.	R.I.	Vt.	Va.	W.Va.	Canada
Consignment Totals		1544	3541	1197	1881	1555	1915	6313	5772	4833	5621	1002	394	4947	482	4323
Respiratory infection (unidentified)	0-4 Wks					1	6		8		a 7			30	1	8
	1-5 Mos				5	2	17		28					42		3
	+ 5 Mos					2	3		19					10	1	2
	Other					T-1			0-9	Pkt-2			0-2			0-75
Riboflavin deficiency	0-4 Wks	a 7	6	2	12	1	6	b 6	5	1	a 11			12	1	
	1-5 Mos			1	1					1				12		
	+ 5 Mos													2		
	Other				T-3											
Rickets & Osteomalacia	0-4 Wks	a 5	10	2	1	2	12	b 21	12	1	a 12			6	1	
	1-5 Mos		5	4		2	2		10	20		3		1	2	1
	+ 5 Mos				1	7			6	20		1		3		
	Other								0-10	T-1	T-7	0-2			T-2	
Synovitis, Infectious	0-4 Wks	a 55	3	2	4		1	b114	2	2	a 107		a19	5		3
	1-5 Mos		95	81	116	41	87		155	136		3		50	17	93
	+ 5 Mos		1	19	16	16	34		53	15		1		22	5	21
	Other	T-2	T-6		T-4	T-1	T-1		0-7	T-18	T-18		T-1		T-9	0-26
Tendon, ruptured (gastrocnemius)	1-5 Mos	a 9		3	1	5	4	b 1	7		a 8	2				4
	+ 5 Mos			2	1	8	11		14			3				4
	Other				0-2											
Teniasis	0-4 Wks	a 7	2					b313			a 133		a 2	2		
	1-5 Mos		9	1	1	6	5		4	47				14		6
	+ 5 Mos		8	7	3	26	15		18	69				29		24
	Other								D-1		D-1					
Trichomoniasis	1-5 Mos		3						3	28	a 3					5
	+ 5 Mos								1	13						6
	Other		T-1		Pgn-3			Pgn-9	0-10	0-3	0-8					0-12
Tuberculosis, Avian	1-5 Mos	a 1									a 5		a 3			2
	+ 5 Mos				7	1			7			1		7		31
	Other													T-1		0-3
Tumors	1-5 Mos	a 1	1	2	1	2	3	b206	21		a 70		a 1			1
	+ 5 Mos			11	10	17	3		117					3	6	10
	Other	0-7		0-2	T-1	0-3			0-5	Pkt-1	0-17	Pkt-1	0-5			0-4
Typhoid, Fowl	0-4 Wks		1					b 30		2	a 33		a 1			
	1-5 Mos		3		3					9				2		1
	+ 5 Mos				16					29				6		5
	Other									T-3						
Xanthomatosis	+ 5 Mos							b 11	2	3	a 8					

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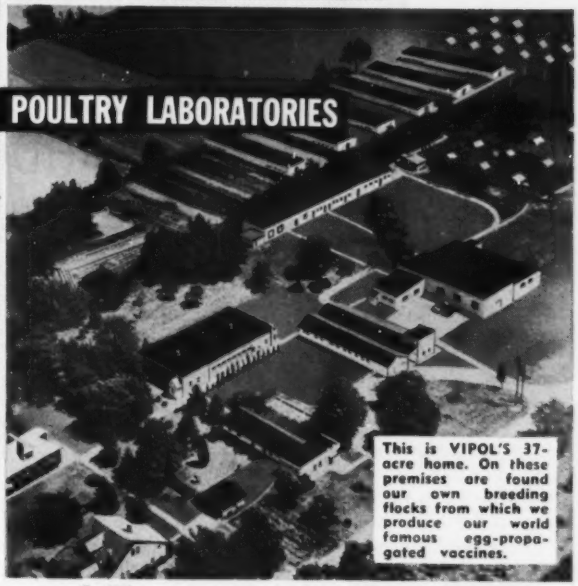

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and for chronic respiratory disease (C. R. D.) of poultry . . .

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1. Johnson, C.: Paper presented at the 45th Annual Meeting of the Poultry Science Association, North Carolina State College, Raleigh, North Carolina, Aug. 7-10, 1956.

2. Shumard, R. F.: Paper presented at the First National Symposium on Nitrofurans in Agriculture, Michigan State University, East Lansing, Michigan, Sept. 28-29, 1956.

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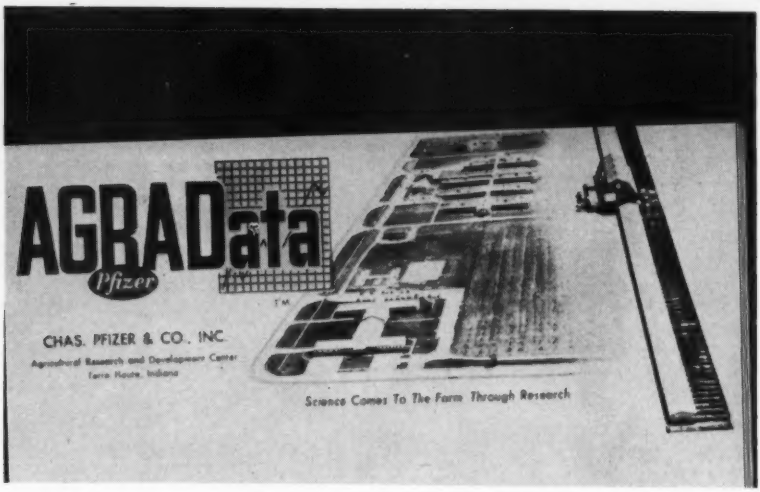

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References: 1. Couch, J. R.: First Conference on the Use of the Tranquilizing and Antihypertensive Agent SERPASIL in Animal and Poultry Production, College of Agriculture, Rutgers, The State University, New Brunswick, N. J., May 7, 1959. 2. Carlson, C. W.: Ibid. 3. Barnett, B. D.: Second Conference on the Use of Reserpine (Serpasil) in Poultry Production, The Institute of Agriculture, University of Minnesota, St. Paul, Minn., May 6, 1960. 4. Carlson, C. W.: Ibid. 5. Waibel, P. E.: Ibid. 6. Morrison, W. D.: Ibid. 7. Weiss, H. S.: First Conference, etc. 8. Burger, R. E.: Ibid. 9. Parker, E. L.: Second Conference, etc. 10. Weiss, H. S.: Ibid.

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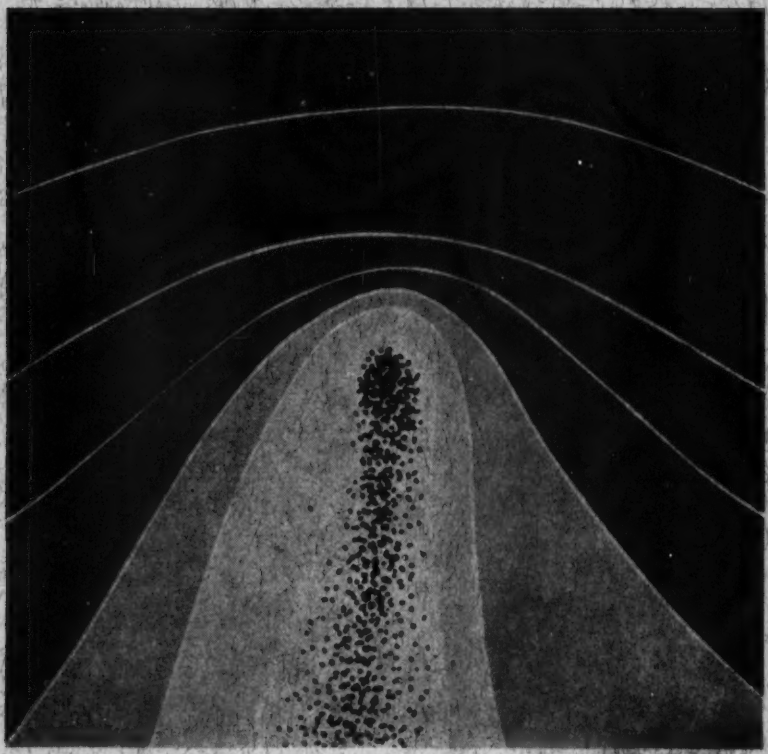

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